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The importance of plant proteins from ABCB subfamily in auxin transport

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Statement

I hereby declare that the work presented in this thesis is my own and was carried out entirely with help of literature and aid cited in the thesis. This thesis is not a subject of any other defending procedure.

Prague, Czech Republic
July 18th, 2011 Mgr. Martin Kubeš

On behalf of the co-authors of the papers published, I hereby confirm the agreement with inclusion of the papers below into the dissertation thesis of Martin Kubeš. The papers were produced as a team work and the particular contribution of Martin Kubeš is specified at the beginning of relevant chapters of the thesis.

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July 18th, 2011 doc. RNDr. Eva Zažímalová, CSc.

Content

Abbreviations

1-NOA	1-naphthoxyacetic acid
2-NOA	2-naphthoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
4-Cl-IAA	4-chloroindole-3-acetic acid
AAP	amino acid permeases
ABC	ATP-binding cassette
ABP1	auxin binding protein 1
ANT	aromatic and neutral amino acid transporter
Arabidopsis	*Arabidopsis thaliana* (L.) Heynh.
ARF	auxin response factor
ATF	amino acid transporter family
ATIs	auxin transport inhibitors
AUX1	AUXIN RESISTANT 1
AuxRE	auxin response element
AVP1	ATPase/ hydrogen-translocating pyrophosphatase (AVP1)
AXR4	AUXIN RESISTANT 4
BeA	benzoic acid
BFA	brefeldin A
BRET	bioluminescence resonance energy transfer
BUM	2-[4-(diethylamino)-2-hydroxybenzoyl] benzoic acid
BY-2	*Nicotiana tabacum* L. cv. Bright Yellow-2 cell suspension culture
CHPAA	3-chloro-4-hydroxyphenylacetic acid
CPD	2-carboxylphenyl-3-phenylpropan-1,3-dione
DEX	dexamethasone, (8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9-Fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one
DMSO	dimethyl sulfoxide
doxorubicin	(8*S*,10*S*)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2*H*-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione
ER	endoplasmic reticulum
EST	estradiol

FKBD	FK506-binding domain
FKBP	FK506-binding protein
FRAP	fluorescence recovery after photobleaching
gravacin	3-(5-[3,4-dichlorophenyl]-2-furyl) acrylic acid
HUGO	Human Genome Organization http://www.hugo-international.org/
IAA	indole-3-acetic acid
IAAld	indole-3-acetaldehyde
IBA	indole-3-butyric acid
LAX	LIKE AUXIN RESISTANT
LHTS	lysine histidine transporters
MDR	multidrug resistance
MRP	multidrug resistance associated
NAA	1-naphthaleneacetic acid
NAA-Glc	NAA glucosyl ester
NBD	nucleotide binding domain
NPA	1-naphthylphthalamic acid
NRT 1.1	nitrate transmembrane transporter
PAT	polar auxin transport
PDR	pleiotropic drug resistance
PGP	P-glycoprotein
P_i	inorganic phosphate
PM	plasma membrane
PPIase	peptidyl-prolyl cis-trans isomerase
ProT	proline transporters
ROS	reactive oxygen species
TIBA	2,3,5-triiodobenzoic acid
TMD	transmembrane domain
TWD1	twisted dwarf 1
verapamil	*(RS)*-2-(3,4-dimethoxyphenyl)-5-{[2-(3,4-dimethoxyphenyl)ethyl]-(methyl)amino}-2-prop-2-ylpentanenitrile
Xanthi	*Nicotiana tabacum* L. cv. Xanthi XHFD8 cell suspension culture

Summary

Polar auxin transport provides essential directional and positional information for many developmental processes in plants. At the cellular level, it is realized by both the passive diffusion and the active transport through the membrane proteins - AUX1/LAXes, PINs and ABCBs. The aim of this thesis was to characterize the role of ABCB1, ABCB4 and ABCB19 proteins in polar auxin transport using transformed tobacco BY-2 cell lines. It was shown that localization of the ABCB1, 4 and 19 is not polar on the plasma membrane (PM). The ABCB4 was also more stable on PM after the treatment with auxin influx inhibitors 1-NOA, 2-NOA and CHPAA; making use of ABCB4-cell line helped to uncover new characteristics of markers of endocytosis – the FM-dyes. The induction of ABCB19 has led to the decrease in ^{3}H-NAA accumulation with characteristic cell phenotype (cells ceased to divide, started to elongate and there was formed an increased amount of starch granules), similar to the PIN7 overexpressing cell line. Thus, also the ABCB-based enhanced auxin efflux resulted in depletion of auxin from cells, and concomitantly changed their developmental program. The auxin starvation phenotype in the cell line with induced overexpression of PIN7 could be rescued by application of the auxin efflux inhibitor NPA; however, the accumulation of auxin in the ABCB19-overexpressing cell line was less sensitive to NPA, and the rescue of the auxin starvation phenotype was ineffective there. Importantly, the unique property of the ABCB4 was demonstrated: It displayed the dual, auxin-concentration-dependent auxin transport activity in *Arabidopsis* roots, tobacco BY-2 and yeast cells. The results also suggested that the non-competitive inhibition of the ABCB4-mediated auxin efflux contributes to the herbicidal effects of the synthetic auxin analogue 2,4-D. Besides intercellular transport, there is another process with the potential to modify auxin level, the metabolism. Auxin metabolic profiles together with data from auxin transport assays were produced, and they allowed mathematical modelling of auxin transport on the cellular level by utilizing real quantitative experimental data. It was shown that NAA is strongly and rapidly metabolized in BY-2 cells and the predominant metabolite was identified as NAA glucosyl ester. This metabolite was retained in cells, thus raising apparent intracellular concentration of NAA previously measured during auxin accumulation experiments. This might lead to serious underestimation of auxin efflux carriers transport capacity for NAA as well as IAA measured in the past. The mathematical modelling using both experimental data on accumulation of auxins together with metabolic profiling is currently in progress.

Souhrn

Polární transport auxinu poskytuje základní informaci o směru a poloze pro řadu vývojových procesů v rostlinách. Na buněčné úrovni je uskutečňován jak pasivní difuzí tak aktivním transportem prostřednictvím membránových proteinů – AUX1/LAX, PIN a ABCB. Hlavním cílem této disertační práce bylo charakterizovat roli ABCB1, ABCB4 a ABCB19 proteinů v polárním transportu auxinu s využitím transformovaných tabákových BY-2 buněčných linií. Ukázalo se, že lokalizace ABCB1, 4 a 19 proteinů na plazmatické membráně je nepolární. Také ABCB4 protein na plazmatické membráně byl po ošetření inhibitory auxinového vtoku do buňky 1-NOA, 2-NOA a CHPAA více stabilní; navíc využití ABCB4 buněčné linie pomohlo odhalit nové vlastnosti endocytotických markerů – FM barviv. Indukce ABCB19 proteinu vedla ke snížení akumulace ^{3}H-NAA s charakteristickým buněčným fenotypem (buňky se přestávaly dělit, začaly se prodlužovat a docházelo k vytváření a zvětšování škrobových zrn), podobně jako u PIN7 overexprimované buněčné linie. Proto také zvýšený výtok auxinu způsobený overexpresí ABCB genů vedl k vyčerpání auxinu z buněk a současně ke změně jejich vývojového programu. Projev fenotypu auxinového hladovění v overexprimované buněčné linii PIN7 mohl být zvrácen aplikací inhibitoru auxinového výtoku NPA, zatímco v případě k NPA méně citlivé ABCB19 overexprimující buněčné linii ke zvrácení tohoto fenotypu nedošlo. Za důležité lze považovat, že se podařilo prokázat jedinečnou vlastnost ABCB4 proteinu: byla naznačena jeho duální koncentračně závislá funkce při transportu auxinu v kořenech *Arabidopsis*, tabákových BY-2 a kvasinkových buňkách. Výsledky také poukázaly na fakt, že nekompetitivní inhibice ABCB4 zprostředkovaného auxinového výtoku přispívá k herbicidním účinkům syntetického auxinového analogu 2,4-D. Kromě mezibuněčného transportu je zde metabolismus jako další proces s potenciálem měnit hladiny auxinu. Získané metabolické profily auxinů společně s údaji z auxinových transportních esejí nám umožnili modelovat auxinový transport na buněčné úrovni s využitím reálných kvantitativních experimentálních dat. Ukázalo se, že na úrovni BY-2 buněk je NAA silně a rychle metabolizována a převládající metabolit byl identifikován jako NAA glukosyl ester. Tento metabolit byl zadržován v buňkách, čímž dříve při auxinových akumulačních experimentech docházelo ke zdánlivému zvyšování naměřené intracelulární koncentrace NAA. To by mohlo vést k vážnému podcenění transportní kapacity auxinových přenašečů pro NAA, ale i IAA u dat naměřených v minulosti. V současné době probíhá matematické modelování využívající experimentální data z akumulací auxinů společně s daty z metabolických profilů.

Nomenclature of ABC genes and proteins

Naming of plant ABC genes proceeded initially on a gene-by-gene basis until the completion of the first drafts of the *Arabidopsis thaliana* and *Oryza sativa* genomes, which led to the publication of several partial and near-complete inventories of plant ABC genes (Sánchez-Fernández et al., 2001a; Martinoia et al., 2002; van den Brûle and Smart, 2002; Jasinski et al., 2003; Garcia et al., 2004; Crouzet et al., 2006; Sugiyama et al., 2006).

Although these publications have proved to be extremely useful, the independent identification of the same genes by several groups and the use of different naming systems by different authors have introduced several conflicting names into the literature. It was confusing to follow the ABC research field and so, together with accelerating completion of plant genome sequencing, the work on unified system of ABC genes nomenclature was initiated.

Verrier et al. (2008) proposed a simple naming system that provides a unique, systematic identifier for each gene. Currently, plant ABC subfamilies are usually named after their human or microbial prototypes (e.g. multidrug resistance (MDR), multidrug resistance associated protein (MRP) and pleiotropic drug resistance (PDR)) as prescribed by Sánchez-Fernández et al. (2001b). Therefore, Verrier et al. (2008) unified plant and animal ABC naming systems and proposed to adopt the Human Genome Organization (HUGO)-approved subfamily designations, which the vertebrate and invertebrate ABC communities used widely (Dean et al., 2001; Dean and Annilo, 2005). Aligning amino acid sequences of NBDs and performing phylogenetic analysis group most eukaryotic ABC proteins into eight major subfamilies (A–H) and prokaryotic ABC proteins into one subfamily (I), regardless of the species of origin. These subfamilies are largely defined by domain organization (configuration of domains in the protein) and content (presence of additional domains; half-size versus full-size transporters).

In this thesis, new nomenclature proposed by Verrier et al. (2008) is used but in some cases usage of old synonyms for ABCB genes like ABCB1/PGP1, ABCB19/MDR1/PGP19 and ABCB4/PGP4 could not be avoided. The “old” names were used mainly in papers published before the year 2008 and in all references from the same period. In those cases all relevant names for given gene/protein are used.

1. Introduction

The plant hormone auxin has a unique position among other plant growth regulatory substances. A wide spectrum of developmental processes in plants is controlled by the differential distribution of its molecules (Benjamins and Scheres, 2008). Moreover, mutual proportion between endogenous auxins and cytokinins is crucial for regulation of plant cell division, elongation and differentiation (Davies, 2004).

Various levels of auxins in plant tissues or organs are created in response to both exogenous stimuli and the internal developmental program, and thus they provide interconnection between environmental or endogenous signals and signalling pathways, resulting in particular developmental events. In principle, there are two processes that have the potential to modify the level of any compound in a cell: metabolism and intercellular transport. Indeed, in the case of auxin, both metabolic changes of auxin (reviewed by Normanly et al., 2010; Ludwig-Müller, 2011) as well as its transport (reviewed by Petrášek and Friml, 2009; Vanneste and Friml, 2009; Zažímalová and Murphy et al., 2010) have been shown to be involved in modulation of plant development.

The native auxin such as indole-3-acetic acid (IAA) mediates interactions between cells, tissues and organs over both short distances (e.g. between adjacent cells) and over long ones (e.g. between the shoot apex and sites of lateral root initiation) (Morris et al., 2004). The auxin cell-to-cell movement contributing to both short and long distance auxin flow is unique compared to other plant hormones; at the cellular level it is realized by both passive diffusion and active translocation across PM and it is mostly polar (Rubery and Sheldrake, 1974; Raven, 1975; Goldsmith, 1977). IAA transport plays a crucial role in the initiation and/or maintenance of cell and tissue polarity and axiality, upon which pattern formation depends (Morris et al., 2004). Generally, auxin determines - mostly via its polar transport - the shape of the plant (Friml, 2003).

The *Arabidopsis thaliana* genome encodes one AUX1 and three Like AUX1 (LAX1, LAX2 and LAX3) proteins (Parry et al., 2001a, b). As mentioned above, auxin molecules can enter cells passively; however, they can also be transported into cells via the H^+ - symport activity of this AUX1/LAX family of PM permeases (reviewed by Kerr and Bennett, 2007).

Previous studies have demonstrated that the polarized movement of auxin is dependent on the action of polarly localized and constitutively cycling PIN auxin efflux proteins (reviewed by Petrášek and Friml, 2009; Friml, 2010). In *Arabidopsis thaliana*, the PIN family consists of eight members and it divides into two basic subclades, differing in the length of a hydrophilic loop localized in the middle of their polypeptide chain (reviewed by Křeček and Skůpa et al., 2009).

Another proteins playing role in auxin influx and efflux are plant orthologs of mammalian ABC transporters from the ABCB (MDR/PGP) protein subfamily (Noh et al., 2001; Geisler et al., 2005). ABCB1 (PGP1) and ABCB19 (MDR1/PGP19) have been shown to act as auxin efflux carriers (reviewed by Geisler and Murphy, 2006; Titapiwatanakun and Murphy, 2009), whereas ABCB4 (PGP4) exhibits more complex auxin transport characteristics. Santelia et al. (2005) and Terasaka et al. (2005) showed that ABCB4 is involved in auxin transport processes controlling lateral root and root hair development in *Arabidopsis thaliana*, and it also functions in the basipetal redirection of auxin flow from the root tip. ABCB4 expression in tobacco BY-2 cells was shown to mediate NAA efflux (Cho et al., 2007), and the authors speculated about ABCB4 efflux activity which reduces auxin levels in the root hair cells and consequently inhibits root hair elongation. On the other hand, heterologous expression of ABCB4 in mammalian HeLa cells resulted in net increase of IAA retention indicating ABCB4 function in auxin uptake (Terasaka et al., 2005). The uptake activity of ABCB4 is not unexpected because it exhibits sequence homology with the berberine alkaloid uptake transporter CjMDR1/ABCB1 from *Coptis japonica* (Shitan et al., 2003). Moreover ABCB4 expression in *Schizosaccharomyces pombe* cells displayed a concentration-dependent reversal of IAA transport (Yang and Murphy, 2009). These facts have pointed to inconsistencies related to auxin transport activity and function of the plant ABCB4 protein.

Beside these PM-localised auxin transporters (AUX1/LAX, PINs and ABCBs) there are other proteins able to transport auxin. For instance, as recently published, *Arabidopsis thaliana* NRT1.1 nitrate transporter (reviewed by Gojon et al., 2011), which acts as a nitrate sensor and is crucial for nitrate signalling governing root growth, not only transports and senses nitrate but it also facilitates auxin uptake (Krouk et al., 2010, 2011). Moreover, nitrate inhibits the NRT1.1-dependent auxin uptake, suggesting that transduction of nitrate signal by NRT1.1 is associated with a modification of auxin transport. Similar properties could be expected also for other anion transporters, such as malate etc. (discussed in Zažímalová and Murphy et al. 2010).

Anyway, in spite of recent fast progress in understanding auxin transport, there are still a lot of open questions related to the mechanism of action of all types of auxin transporters and their role in plant development.

2. Outlines of the thesis

The first reviews touched the problematics of plant ABC proteins from the side of knowledge about their animal or human counterparts (Dudler and Hertig, 1992; Higgins, 1995). The main inspiration came from the multidrug resistance protein (MDR1), which was connected with a phenomenon described in mammalian tumor cells and their resistance to chemotherapeutics where tumor cells initially sensitive to a cytotoxic drug could evolve into a state in which they were not only resistant against the same drug but also against a wide variety of structurally unrelated drugs (reviewed by Endicott and Ling, 1989; Van der Bliek and Borst, 1989; Pastan and Gottesman, 1991). First information about plant ABC proteins structure and function were published at the beginning of this century (Davies and Coleman, 2000; Theodoulou, 2000; Sánchez-Fernández et al., 2001a, b; Martinoia et al., 2002; Rea et al., 2002). Simultaneously, Jasinski et al. (2003) and Garcia et al. (2004) indicated phylogenetic relationship between *Arabidopsis thaliana* and *Oryza sativa* ABC genes inside three main subfamilies (*ABCB/MDR*, *ABCC/MRP* and *ABCG/PDR*). *Arabidopsis thaliana* proteins from ABCC/MRP and ABCG/PDR subfamilies were more minutely described by Kolukisaoglu et al. (2002) and van den Brûle and Smart (2002).

In the collaboration with the laboratory of Dr. Jiří Friml, I have started the work by the successful transformation of inducible constructs carrying *ABCB1* and *ABCB19* genes into BY-2 cells. After the western blot verification of recombinant protein production and cell phenotype characterization I used these cell lines for auxin accumulation experiments. Results are shown in Chapter 1 of this thesis and published in Petrášek et al. (2006).

Besides the auxin accumulation experiments I have also tested some inhibitors of ABC proteins used in microbiology and human biology (like doxorubicin, vanadate, verapamil). Vanadate was shown to inhibit ATPase activity of ABC transporter for maltose (MalFGK2) of *Salmonella typhimurium* (Hunke et al., 1995) or ABC transport complex in *Escherichia coli* (Davidson et al., 1996). Later, Fetsch and Davidson (2002) described that vanadate catalyzes photocleavage of the signature motif LSGGQ and the nucleotide-binding (Walker A) motif of ABC maltose transport complex in *Escherichia coli*. Consecutively Terasaka et al. (2003) showed that vanadate-induced nucleotide trapping technique could be applicable to plant cells for characterizing ABC proteins expressed in berberine-producing cell cultures (*Thalictrum minus* and *Coptis japonica*). Loe et al. (2000) demonstrated that verapamil stimulates glutathione transport by MRP1 in tumor cells. Correspondigly, multidrug resistance is the most widely studied manifestations of tumor cells resistance to doxorubicin (Bradley et al., 1988). My goal was according to already mentioned drug effects to interfere with the intracellular localization or

the activity of ABCB19. Unfortunately, on the level of cell phenotype and viability no significant differences between treated and untreated cells were observed.

While ABCB1 and ABCB19 inducible lines of BY-2 were not stable, for later experiments it was necessary to repeatedly transform new BY-2 cells and work with freshly established lines. The other set of results that is presented in Chapter 2 of this thesis and published in Mravec et al. (2008) shows the phenotypical comparisons between inducible lines carrying various PIN genes with ABCB19 cells.

Apart from new BY-2 cell transformations I started together with Dr. Klára Hoyerová the accumulation assays in short and long stems (inflorescence) segments of *Arabidopsis thaliana* plants transformed with inducible *ABCB1* and *ABCB19* genes. We used partially modified methods of Goldsmith (1982) and Parry et al. (2001a). The importance of ABCB1 and ABCB19 for long-distance auxin transport in *Arabidopsis* plants was showed, while in induced ABCB1 and ABCB19 short stem segments the ^{3}H-NAA retention was enhanced comparing to wild-type control and non-induced variants. Moreover both used inhibitors gravacin and NPA increased net ^{3}H-NAA retention. Preliminary results were presented as a poster at the ACPD conference in Prague 2009.

As the continuation of my effort to address the auxin transporting role of ABCB proteins, I have moved my interest to the functionaly enigmatic ABCB4 protein. Despite several contrary results about ABCB4 auxin transport directionality (Terasaka et al., 2005; Santelia et al., 2005; Cho et al., 2007) together with indication of possible ABCB4 dual transport function (Yang and Murphy, 2009), nobody has provided conclusive evidence on the auxin transport directionality through this protein. I started with transformation of BY-2 cells with *ABCB4-YFP* from Dr. Hyung-Taeg Cho, but it was not successful. Therefore, I have contacted Dr. Angus Murphy for providing me with *ABCB4-GFP* gene construct under native and constitutive promoter. Transformation with this construct was successful and the localization of ABCB4 was characterized in terms of its membrane stability and helped to uncover new characteristics of FM endocytic dyes (Chapter 3 of this thesis; Jelínková and Malínská et al., 2010) as well as auxin influx inhibitors 1-NOA, 2-NOA and CHPAA (Chapter 4 of this thesis; Laňková et al., 2010). Concurrently I have initiated first ABCB4 auxin accumulation experiments. On ACPD conference in Prague 2009 I discussed with Dr. Angus Murphy my actual results and we started collaboration on ABCB4 dual transport function. We continued on BY-2 cell level and they contributed with *Arabidopsis thaliana* roots data and data from yeast IAA accumulation, where they confirmed ABCB4 concentration dependent dual transport function. Results are presented in the Chapter 5 of this thesis and manuscript is resubmitted in the Plant Journal.

Before starting ABCB4 story, I was welcomed to the project of my colleague Sibu Simon, where he was interested in the action of auxin and auxin analogues. The manuscript is submitted in the Chemistry&Biology journal. This manuscript is not included in submitted thesis but it is available electronically on the attached CD together with my contribution and specification of my results. During this project we were suspicious about the contamination or degradation of our radiolabeled auxins stocks. Thus I have started fruitful collaboration with Dr. Petre Dobrev. He did analysis of ^{3}H-2,4-D, ^{3}H-IAA and ^{3}H-NAA stocks used also by other colleagues in the lab and confirmed our suspicious in case of ^{3}H-IAA, where nearly 35% of IAA itself was degradated, in case of ^{3}H-NAA and ^{3}H-2,4-D it was about 5%. Later I found out that the reason for ^{3}H-IAA degradation was coming from the side of our suppliers and instability of this compound. From the auxin purity analysis on HPLC system, it was only small step to the routine metabolic profiling in cell cultures and plants.

Based on the ^{3}H-2,4-D, ^{3}H-IAA and ^{3}H-NAA metabolic profiles in BY-2 cells and cultivation media it was soon very clear that it is not possible to separate transport and metabolism, but it is necessary to think about both processes together. Moreover, combination of results from accumulation and metabolism in BY-2 cells allowed us to prepare mathematical model of auxin transport on the cellular level. This project was great challenge for all team members because it required teamwork between biologists and mathematicians. Our joint effort is presented in emerging interdisciplinary manuscript, presented as Chapter 6 of this thesis.

3. Objectives of the thesis

The main objective of this thesis was to contribute to understanding the role of ABCB1, ABCB4 and ABCB19 proteins (members of ATP-binding cassette (ABC) family of transporters) in polar auxin transport.

Particular objectives were:

1) To clarify the role of these proteins in polar auxin transport and to determine their intracellular localization.
2) To reveal possible interactions between ABCB and PIN proteins in plants.
3) To understand the kinetics of auxin transport by the ABCB4 protein.
4) To determine auxin metabolic profiles in tobacco BY-2 and Xanthi cell cultures as one of the prerequisites for the creation of mathematical model of auxin transport on the cellular level.
5) To optimize and assess the method of extraction and purification, suitable for determination of auxins by means of HPLC-tandem mass spectrometry, including identification of the main auxin metabolites in BY-2 cell culture.

4. Literature overview

4.1. The plant growth regulator - auxin

Although plants do not have typical animal long distance signalling mechanisms like nervous system, they developed alternative ways to establish cell-to-cell communication. One of them is transport of plant hormone auxin which represents an outstanding intercellular communication system in plants. Polarized auxin transport provides essential directional and positional information for developmental processes, such as vascular differentiation, apical dominance, patterning, organ polarity, embryogenesis, organogenesis, phyllotaxis or tropisms (Benková et al., 2003; Blancaflor and Masson, 2003; Blilou et al., 2005; Pekker et al., 2005; Weijers and Jurgens, 2005). Disruption of directional auxin movement by genetic or pharmacological manipulations results in severe developmental defects (reviewed by Friml, 2003). Although a detailed understanding of the molecular basis for the complexity of auxin activity has not yet emerged, progress during the past 15 years has led to identification of critical components of auxin signalling and provided a framework for addressing how auxin regulates diverse developmental processes.

4.2. Historical overview

The idea of plant hormones initially originated in the 19th century as Julius von Sachs supposed that plant organ-forming substances move directionally within the plant, including a root-forming substance produced in leaves that moves downward (Sachs, 1868). Concurrently Charles Darwin in his work "The power of movement in plants" described the phototropic experiments with canary grass (*Phalaris canariensis* L.) coleoptiles, and suggested the existence of a mobile substance which transmits the light sensing at the tip of the coleoptiles into differential growth at the base of the coleoptiles (Darwin, 1880). Five years later Salkowski isolated indole-3-acetic acid (IAA) from fermentation media (Salkowski, 1885) but the concept of the hormone itself, its isolation, purification from plants and characterization took another 50 years (Went, 1926; Went and Thimann, 1937). Went used small agar blocks on which he placed the coleoptiles tips of oat (*Avena sativa* L.) stems. The compound accumulated in the agar block resumed the growth of decapitated stems. Kogl and Haagen-Smit (1931) called the new compound an auxin. This name comes from the Greek word "auxein" which means to grow.

4.3. Auxin metabolism – biosynthesis, conjugation and catabolism

A key missing link in our understanding of auxin-regulated developmental processes in plants is that the molecular mechanisms of auxin biosynthesis are not known completely.

Without this knowledge it is difficult to understand the exact mechanism of auxin movement and how auxin gradients are created and maintained. The most abundant form of native auxin is IAA but plants produce also indole-3-butyric acid (IBA) (Cooper, 1935), phenyl acetic acid (PAA) (Koepfli et al., 1938) and 4-chloroindole-3-acetic acid (4-Cl-IAA) (Porter and Thimann, 1965). Because of the relative instability of natural auxins, the stable synthetic auxin analogues 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are used in most of the experiments (reviewed by Woodward and Bartel, 2005).

The synthesis of auxin in *Arabidopsis thaliana* occurs mainly in leaves, cotyledons and roots (Ljung et al., 2001; Ljung et al., 2005). Although multiple pathways including three tryptophan-dependent routes (with indole-3-pyruvic acid, indole-3-acetamide, indole-3-acetaldoxime, and tryptamine as main intermediates) and one tryptophan-independent route (with either indole-3-glycerol or indole as auxin precursors) are proposed to synthesize auxin in plants, the molecular details and the physiological roles of each pathway are not known (Bartel, 1997; Cohen et al., 2003; Woodward and Bartel, 2005). Also due to the high functional redundancy of auxin biosynthetic genes and the complexity of auxin synthesis it is difficult to fully characterize these pathways (Fig. 1; reviewed by Woodward and Bartel, 2005).

Research of auxin biosynthesis in plants appears to be very difficult, and no auxin-deficient mutant has ever been identified. However, there are several auxin overproduction *Arabidopsis thaliana* mutants including *yucca* (Zhao et al., 2001), *sur1* (Boerjan et al., 1995; King et al., 1995), *sur2* (Delarue et al., 1998), or overexpressing lines CYP79B2 and CYP83B1 (Delarue et al., 1998; Barlier et al., 2000; Zhao et al., 2002).

All three tryptophan-dependent pathways converge at indole-3-acetaldehyde (IAAld), which is oxidized by IAAld oxidase to IAA. Interestingly, an aldehyde oxidase is also involved in the final step of abscisic acid biosynthesis. IAAld oxidase activity has been measured in plants, and the small aldehyde oxidase gene family in *Arabidopsis thaliana* has been described (Sekimoto et al. 1998; Seo et al., 1998).

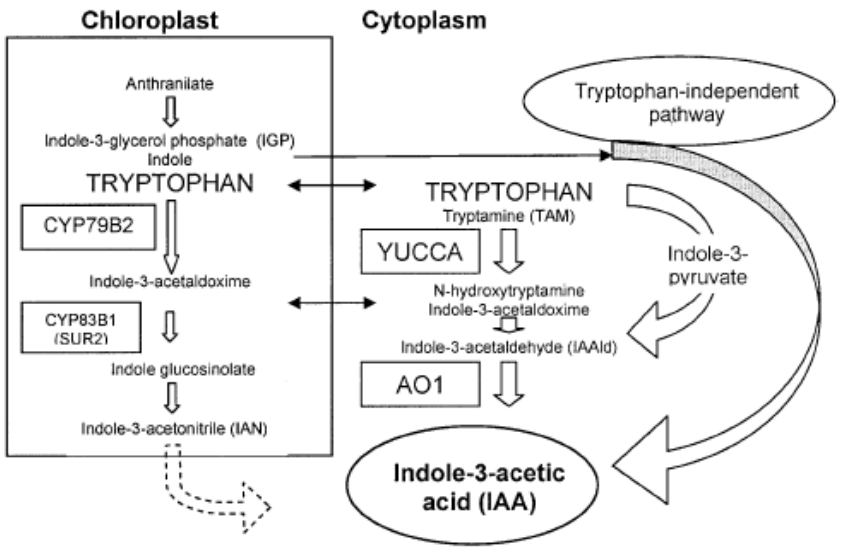


Figure 1. Parallel pathways for auxin biosynthesis
The dashed arrow illustrates the glucosinolate pathway restricted to few plant families. The long arrow on the right indicates the non-described tryptophan-independent pathway (adopted from Zažímalová and Napier, 2003).

Auxin conjugation and catabolism are other important players in auxin metabolism beside biosynthesis. Generally, active phytohormones are changed into multiple forms by range of chemical reactions like acylation, esterification or glycosylation. It seems that conjugated compounds can serve as a pool of inactive forms that can be converted to active forms by de-conjugation reactions. The concept of reversible conjugation of auxins suggests that under changeable conditions these compounds can be a source of free hormones. Auxin catabolism results in a loss of activity and decreases the size of the auxin bioactive pool. All catabolic steps are in principle irreversible, except for some processes such as the formation of ester, glucoside and amide conjugates in some plants (reviewed by Woodward and Bartel, 2005; Bajguz and Piotrowska, 2009; Ludwig-Müller, 2011).

For example, conjugation of IAA with amino-acids or sugars can follow after its synthesis probably as the way of storage or inactivation. IAA can be released after oxidation or hydrolysis of its conjugates (reviewed by Cohen and Bandurski, 1982). Free IAA is biologically inactivated by oxidation (e.g. to 2-oxindole-3-acetic acid) or by conjugation. Some of IAA conjugated to indole-3-acetyl-*N*-aspartic acid is further catabolized; however, conjugates are also used as storage compounds. IAA is conjugated in a variety of ways – as amides to amino acids, peptides and proteins, and to sugars through both ester and N-linkages. Conjugate hydrolysis releases free IAA, and such hydrolysis is the principal source of auxin in germinating seeds. It seems that conjugates are also transported within the plants (Zažímalová and Napier, 2003).

The molecular details (e.g. molecular mechanisms of auxin biosynthesis, conjugation and degradation together with the action of particular enzymes), physiological roles of each biosynthetic pathway and evolutionary context are the subjects of intensive study (reviewed by Cooke et al., 2002; Normanly 2010; De Smet et al., 2011; Ludwig-Müller 2011).

4.4. Auxin signalling

Proper auxin signalling is indispensable for plant growth and development (reviewed by Santner et al., 2009; Santner and Estelle, 2009) and in plant cells auxin triggers specific genomic and non-genomic responses. The identification and characterization of plant mutants defective in response to auxin led to the disclosure of downstream components of genomic auxin signalling (reviewed in Mockaitis and Estelle, 2008; Calderon-Villalobos et al., 2010). It turned out, that regulated protein degradation is the main factor in auxin transcriptional regulation (reviewed by Paciorek and Friml, 2006). Genome-wide studies indicate that the transcriptional response to auxin is rapid and broad, influencing the expression of a large and diverse set of genes within minutes (Tian et al., 2002; Goda et al., 2004; Overvoorde et al., 2005; Nemhauser et al., 2006).

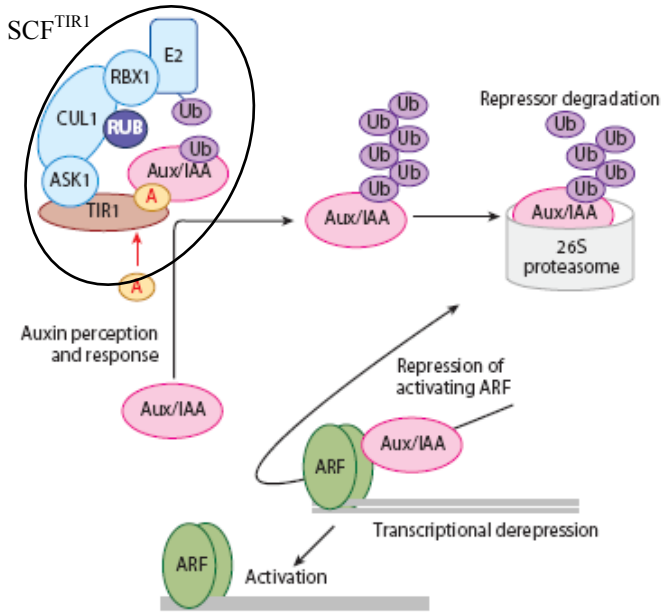


Figure 2. Auxin regulates transcription by promoting ubiquitin (Ub)-mediated degradation of Aux/IAA repressors
Auxin (A) binds to the F-box protein TIR1 in SCF^{TIR1} and stabilizes the interaction between TIR1 and an Aux/IAA substrate. The repressor is polyubiquitinated and degraded by the 26S proteasome. Loss of the Aux/IAA permits auxin response factor (ARF)-dependent transcription of auxin regulated genes. E2, (Ub)-conjugating enzyme (adopted from Mockaitis and Estelle, 2008).

TIR1 F-box protein is the auxin receptor (Dharmasiri et al., 2005a, b; Kepinsky and Leyser, 2005) which forms SKP1-like protein and CULLIN1 into the E3 ligase complex known as SCF^{TIR1} (Fig. 2; Gray et al., 1999; reviewed in Mockaitis and Estelle, 2008; Calderon-Villalobos et al., 2010). After auxin binding to TIR1, this complex promotes substrate ubiquitination and following proteasome-based degradation. The specific targets for this complex are Aux/IAA proteins (Conner et al., 1990; Abel et al., 1995; Gray et al., 2001). Aux/IAA proteins serve as the repressors of transcription factors called auxin response factors (ARFs) (Tiwari et al., 2001; Tiwari et al., 2003). After degradation of the Aux/IAA, the free ARFs can promote the transcription from specific promoters containing small conservative sequence TGTCTC called auxin response elements (AuxRE) (reviewed by Guilfoyle and Hagen, 2007). The huge amount of possible specific interactions among Aux/IAAs (29 genes) and ARFs (23 genes) suggest the way how autonomous auxin responses are mediated (reviewed in Weijers and Jurgens, 2004; Weijers et al., 2005).

However, auxin signalling does not include only transcriptional (genomic) regulation. For example, auxin efflux from the cells is also regulated via inhibition of endocytosis of auxin efflux carriers (and also other PM proteins) by unknown auxin-dependent and BIG-protein-dependent pathway (Paciorek et al., 2005).

A possible component of non-genomic auxin signalling is auxin binding protein 1 (ABP1), which was for the first time detected in membrane fractions from maize (*Zea mays* L.) etiolated coleoptiles (Hertel et al., 1972; Venis, 1977a, b). Later data revealed that ABP1 is targeted to ER and extracellular matrix (Leblanc et al., 1999; Bauly et al., 2000). Other studies demonstrated that ABP1 is involved in auxin binding at the PM and consecutively initiates a transduction pathway, including activation of the proton pump ATPase, acidification of the extracellular space and activation of inward rectifying K^+ channels (reviewed in Goldsmith,

1993; Timpte, 2001; Napier et al., 2002). Interestingly, mutant in *ABP1* gene in *Arabidopsis thaliana* is embryo lethal (Chen et al., 2001a), which suggests its important function in development. Recently, the function of ABP1 in auxin mediated cell division was proposed (David et al., 2007), but the precise role and the involvement in auxin signalling is still unclear. Tromas et al. (2010) reviewed the role of ABP1 in mediating growth and developmental responses together with phylogenetic analysis which revealed that ABP1 is an ancient protein that was already present in various algae and has acquired a motif of retention in ER only recently. Recently, Robert and Kleine-Vehn et al. (2010) demonstrated that ABP1 mediates a nontranscriptional auxin signalling that regulates the evolutionarily conserved process of clathrin-mediated endocytosis and they suggested that this signalling may be essential for the developmentally important feedback of auxin on its own transport.

4.5. Auxin transport - physical and chemical background

To perform its signalling function in plant, auxin must be spatio-temporally distributed and establishment of auxin concentration gradients depends on a tightly regulated transport. Long-distance auxin transport may be typically split in two ways: rapid auxin translocation through vascular system from source tissues to the roots and carrier mediated, slower cell-to-cell polar auxin transport (PAT) which mainly contributes to formation of auxin gradients in meristems (reviewed by Tanaka et al., 2006). The transport of auxin at the cellular level is realized by both passive diffusion and actively through the membrane proteins known as auxin carriers.

The key to understanding how auxin can move across the PM between two adjacent cells in polar orientation lies in the physical-chemical nature of auxin molecules and it was proposed by chemiosmotic hypothesis (Rubery and Sheldrake, 1974; Raven, 1975; Goldsmith, 1977). Because all native auxins are weak acids with amphipathic properties, their ability to penetrate through the PM depends on pH. In acidic apoplast the pH is approximately 5.5 as a result of protons extruded by PM H^+-ATPases and thus only non-dissociated and relatively lipophilic auxin molecules can passively enter the cell by diffusion across the PM. In the neutral cytoplasm (pH = 7.0) almost all auxin molecules are dissociated and trapped inside the cell, and they must be actively exported from the cell by specialized efflux carriers. The asymmetric distribution of auxin efflux carriers then can establish the polarity of auxin flow (Fig. 3; recently reviewed by Zažímalová and Murphy et al., 2010). This hypothesis was tested in the experiment, where decreased apoplastic pH in *Arabidopsis thaliana* overexpressing H^+- pyrophosphatase *AVP1* resulted in increased auxin transport (Li et al., 2005).

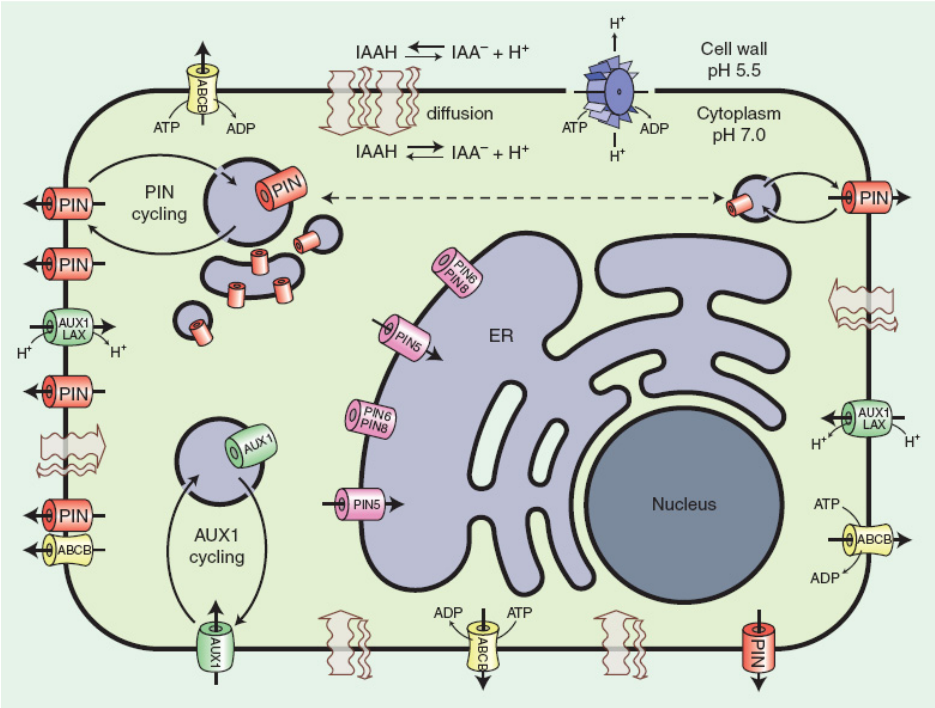


Figure 3. The scheme shows the organization of proteins involved in auxin transport PIN efflux carriers depicted in red represent "long" PINs (PIN1-4, 7), whereas PINs marked in pink represent "short" PINs (PIN5-6, 8) (see below). ER marks endoplasmic reticulum, pale blue structures represent ER and endosomes, curved bold full arrows show constitutive protein cycling, and dashed arrows symbolize the process of transcytosis. Possible collaboration between ABCBs and PINs is suggested by placing the symbols close to each other (adopted from Zažímalová and Murphy et al., 2010).

4.6. Auxin transporters

The previously described physical-chemical background implies the need for active transport of auxin out from the cells. Surprisingly, there are at least two protein families, members of which possess auxin-exporting activity. These are from the plant-specific PIN family (recently reviewed by Křeček and Skůpa et al., 2009, Petrášek and Friml, 2009) and from the B subfamily of ABC transporters (reviewed by Geisler and Murphy, 2006, Titapiwatanakun and Murphy, 2009). PINs are gradient-driven secondary transporters (carriers) while ABCBs are ATP-driven transporters (pumps). As mentioned previously, auxin molecules can enter cells passively; however, they can also be transported into cells via the H^+ symport activity of the AUX1/LAX family of PM permeases (reviewed by Kerr and Bennett, 2007).

The activities of the auxin influx and efflux carriers were described by measuring the cellular accumulation of ^{3}H-IAA, ^{3}H-NAA and ^{14}C-2,4-D in tobacco cell culture (*Nicotiana tabacum* L. cv. Xanthi XHFD8) (Delbarre et al., 1996) or in tobacco protoplasts (Delbarre et al., 1994). It was shown that NAA enters cells by passive diffusion and has its accumulation level controlled by the efflux carrier. In contrast, 2,4-D uptake is mostly driven by the influx carrier but, at least in tobacco cells, 2,4-D is a poor "substrate" for auxin-efflux carriers (Delbarre et al., 1996). Both auxin carriers, as well as passive diffusion into cells contribute to IAA accumulation. The relative contributions of diffusion and carrier-mediated influx and efflux to the membrane transport of 2,4-D, NAA and IAA have been quantified, and the data indicate that plant cells are able to modulate their auxin content by modifying the activity of each carrier (Delbarre et al., 1996).

4.6.1. Auxin influx carriers – AUX1/LAX family

Auxin influx carriers (AUX1/LAX), which mediate the uptake of auxin into the cell, belong to the ATF (amino acid transporter) family of proteins contain five distinct subclasses, AUX1/LAX, the amino acid permeases (AAP), the lysine histidine transporters (LHTs), the proline transporters (ProT) and the new aromatic and neutral amino acid transporter class (ANT) (Young et al., 1999; Ortiz-Lopez et al., 2000). The *Arabidopsis thaliana* genome encodes one AUX1 and three Like AUX1 (LAX1, LAX2, and LAX3) proteins, which amino acid sequence similarity is approximately 80% (Parry et al., 2001b). Chen et al. (2001b) reported that expression of ANT1 in yeast cells facilitates the uptake of both 2, 4-D and IAA, indicating its potential role also as an auxin carrier.

The first identified mutation in auxin influx carrier at the molecular level was presented by Maher and Martindale (1980) who identified the *aux1* mutation in screen for *Arabidopsis thaliana* seedlings less sensitive to 2,4-D. Later AUX1 was characterized at the molecular level in *Arabidopsis thaliana* by Bennett et al. (1996) and its transport function was confirmed by Yang et al. (2006). The *aux1* mutants exhibit reduced root gravitropism and decreased IAA transport in roots and young leaf primordia; further, *aux1* mutants are resistant to inhibitory concentrations of IAA and 2,4-D, but not to more lipophilic NAA (Marchant et al., 1999; Swarup et al., 2001). Because NAA diffuses into cells easily and it is a good substrate for auxin efflux carriers, gravitropic growth in *aux1* mutants is restored by treatment with NAA, but not with 2,4-D, which is taken up only actively - presumably via AUX1 - and it is a poor efflux carrier substrate (Marchant et al., 1999). The growth phenotypes of *aux1* mutants are phenocopied by treatment with the auxin influx inhibitors 1-NOA and CHPAA, which do not affect polar auxin efflux or sensitivity to NAA (Parry et al., 2001a). AUX1 functions in both root basipetal and acropetal auxin transport in a phloem-based auxin transport stream (Swarup et al., 2001). Consistent with this, AUX1 exhibits a basal PM localization in root protophloem cells, where it appears to function in conjunction with apically localized efflux carriers (Marchant et al., 1999; Swarup et al., 2001). Kramer and Bennett (2006) indicated that AUX1 together with other auxin efflux carriers plays a major role in redirection of polar auxin stream in the lateral root cap. Although AUX1 exhibits polar localization on the apical PM in some cells, in others, AUX1 exhibits a non-polar membrane distribution and is also accumulated at the Golgi apparatus and endosomal compartments. The connections between these PM and intercellular localizations have been shown to be dependent on actin filaments and the membrane sterols (Kleine-Vehn et al., 2006). Moreover, the apical localization of AUX1 on the PM of protophloem and epidermal cells requires the presence of AUXIN RESISTANT4 (AXR4), which is found in the ER (Dharmasiri et al., 2006).

Recently Laňková et al. (2010) examined the mechanism of action of the auxin influx inhibitors 1-naphthoxyacetic acid (1-NOA), 2-naphthoxyacetic acid (2-NOA) and 3-chloro-4-hydroxyphenylacetic acid (CHPAA) in transgenic BY-2 cell cultures. The mode of action of these inhibitors has been shown to be linked with the dynamics of the PM. The most potent inhibitor, 1-NOA, blocked the activities of both auxin influx and efflux carriers, whereas 2-NOA and CHPAA at the same concentration preferentially inhibited auxin influx. Laňková et al. (2010) also highlighted the importance of the rate of the particular vesicle trafficking process that may determine the extent of 1-NOA, 2-NOA, and CHPAA action on the PM dynamics.

The importance of AUX1/LAX carriers could be clearly demonstrated in many developmental processes. They are involved in embryogenesis (Ugartechea-Chirino et al., 2010), hypocotyl apical hook development (Vandenbussche et al., 2010), hypocotyl phototropism (Stone et al., 2008), root gravitropism (Bennett et al., 1996), lateral root development (Swarup et al., 2001; Swarup and Benková et al., 2008), root hair development (Jones et al., 2009), phloem loading and unloading (Marchant et al., 2002), and phyllotaxis (Bainbridge et al., 2008). Generally, the importance of auxin uptake carriers lies mainly in their role in the pumping of auxin against its concentration gradient. Mathematical modelling further supports the role of AUX1/LAX proteins in the creation of local auxin maxima as demonstrated for phyllotaxis (Smith et al., 2006).

4.6.2. Auxin efflux carriers - PIN family

The PIN-FORMED (PIN) proteins are plant specific secondary transporters (carriers) acting mostly in the efflux of auxin outside from cells. They are asymmetrically localized within cells and their polarity determines the directionality of intercellular auxin flow.

The first auxin efflux carriers were identified on the base of several *Arabidopsis thaliana* mutants: *agravitropic 1* (*agr1*) (Bell and Maher, 1990), *wavy roots 6* (*wav6*) (Okada and Shimura, 1990), *ethylene insensitive root 1* (*eir1*) (Roman et al., 1995), *pin-formed1* (*pin1*) (Okada et al., 1991). Later was proved, that PIN1 is a plant specific protein with characteristic structure (Gälweiler et al., 1998) and *AGR1*, *WAV6*, *EIR1* genes encode a homologous protein PIN2 (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). Okada et al. (1991), Müller et al. (1998) and Benková et al. (2003) confirmed that all these phenotypes can be phenocopied by exogenous application of auxin efflux inhibitor NPA.

In *Arabidopsis thaliana*, the PIN family consists of eight members and divides into two basic subfamilies according to the length of a hydrophilic loop in the middle of their polypeptide chain.

The “long” PINs (PIN1-4, and 7) are characteristic with the distinct central hydrophilic loop separating two hydrophobic domains (reviewed by Tanaka et al., 2006; Vieten et al., 2007; Křeček and Skůpa et al., 2009). The PIN1-4 and PIN7 proteins act as auxin efflux carriers and are localized at the PM where their polar localization determines the direction of auxin flow (Petrášek et al., 2006; Wisniewska et al., 2006). These “long” PINs have roles in many auxin-dependent processes in plant development (Luschnig et al., 1998; Friml et al., 2002a; 2002b; Blilou et al., 2005; Scarpella et al., 2006; Sauer et al., 2006; Xu et al., 2006). They do not stay statically in their PM domains, but undergo constitutive cycling between the PM and endosomal compartments (Geldner et al., 2001; Dhonukshe et al., 2007). The cycling is relatively fast between various parts of the cell using transcytosis-like mechanism (Kleine- Vehn et al., 2008). The polar localization of the “long” PINs and directional auxin flow are required for embryo development, organogenesis, tropisms, and other developmental processes (Friml et al., 2002b; Friml et al., 2003; Benková et al., 2003; Reinhardt et al., 2003; Blakeslee et al., 2005).

The “short” PINs (PIN5-6 and PIN8) have their central hydrophilic loop either partly (PIN6) or significantly (PIN5 and PIN8) reduced (Křeček and Skůpa et al., 2009). PIN5 has been proved as a functional auxin transporter and its localization on the ER suggests a role in intracellular auxin distribution or regulation of cellular auxin homeostasis, thus controlling availability of active auxin for various subcellular and cellular actions (Mravec et al., 2009). However, even though Mravec et al. (2009) showed PIN5 and PIN8 localization in ER, Ganguly et al. (2010) proposed that PIN8 also localizes in the PM. They showed that PIN8 catalyzes auxin efflux from tobacco BY-2 cells, that PIN8-GFP signal overlaps with FM4-64-labeled plasma membranes in *Arabidopsis thaliana* root hair and tobacco BY-2 cells (while the PIN5-GFP signal does not), that BFA causes the formation of PIN8-containing internal compartments both in *Arabidopsis thaliana* root hair and tobacco cells, and that PIN8 targets to the cell plate in dividing cells (while ER-localized PIN5-GFP does not). So, the mode of action of „short“ PINs is not fully understood yet.

PIN homologs in other plants have also been identified (Křeček and Skůpa et al., 2009), and some of them have been functionally characterized (reviewed by Petrášek and Friml, 2009).

4.6.2.1. Regulation of PIN function and polar targeting

The formation of auxin gradients depends mainly on the regulation of direction of auxin flow by differential AUX1/LAX, PIN and ABCB PM targeting. All of these transporters have been shown to be constitutively recycled between the PM and endosomal compartments.

PINs activity inside the cells is regulated at different levels: transcription (Blilou et al., 2005; Vieten et al., 2005), protein degradation (Abas et al., 2006), protein

phosphorylation/dephosphorylation (Friml et al., 2004; Lee and Cho, 2006; Michniewicz et al., 2007; Zhang et al., 2010) and vesicle trafficking (Steinmann et al., 1999; Geldner et al., 2001, 2003; Paciorek et al., 2005).

There are two possible regulators of the PIN polar localization, the Ser/Thr protein kinase PINOID (PID; Christensen et al., 2000; Benjamins et al., 2001; Friml et al., 2004; Sukumar et al., 2009) and the protein phosphatase 2A (PP2A; Zhou et al., 2004; Michniewicz et al., 2007). High levels of PIN phosphorylation caused by overexpression of *PID* or inhibition of *PP2A* lead to a preferential apical PIN targeting, whereas low phosphorylation levels in the *pid* mutants result in a preferential basal PIN targeting (Friml et al., 2004; Treml et al., 2005). Importantly, PID has been shown to directly phosphorylate the hydrophilic loop of PIN proteins while PP2A phosphatase has been shown to antagonize this (Michniewicz et al., 2007).

PIN proteins seem to be delivered originally in a non-polar orientation after *de novo* synthesis, and their polarity is established by internalization from the PM and polar recycling (Dhonukshe et al., 2008). Therefore, the secretion, clathrin-dependent endocytosis (Dhonukshe et al., 2007), and subsequent recycling are important processes in the PIN polar localization. On the contrary, in dividing cells, PIN proteins are delivered to the forming cell plate by the microtubule-dependent pathway (Geldner et al., 2001; Dhonukshe et al., 2006).

PIN proteins cycle continuously between endosomal compartments and the PM, it means exocytotically to the PM after *de novo* synthesis and endocytotically from PM to the endosomal compartments (Vanneste and Friml, 2009). The exocytosis requires the activity of GNOM protein, an endosomal ADP-ribosylation factor GTPase guanine nucleotide exchange factor (ARF-GEF) (Steinmann et al., 1999; Geldner et al., 2001; Richter et al., 2007), whereas endocytosis occurs in a clathrin-dependent manner (Dhonukshe et al., 2007) and depends on the sterol composition of the PM (Willemsen et al., 2003; Men et al., 2008), which also influences AUX1 (Kleine-Vehn et al., 2006) or ABCB trafficking (Titapiwatanakun et al., 2009; Titapiwatanakun and Murphy, 2009). GNOM seems to be more crucial for basal polar targeting, because the apical PM localization of PINs and AUX1 is not strongly affected when GNOM function is inhibited by BFA (Kleine-Vehn et al., 2008). SORTING NEXIN1 (SNX1) is another component which is involved in regulation of PIN1 cycling (Jaillais et al., 2007).

4.6.3. Auxin influx and efflux carriers – ABCB family

4.6.3.1. The ABC proteins – their discovery, structure and transport function

As it was already mentioned, discovery of ABC proteins is historically connected with the cancer research and with the quest for mechanism of multidrug resistance noticed in various cellular models. The ATP-binding cassette (ABC) proteins are members of the largest protein

superfamily with representatives in all phyla from prokaryotes to eukaryotes (Ames et al., 1992; Higgins, 1992; Rea, 2007; Rees et al., 2009). ABC proteins use the binding and hydrolysis of ATP to power the translocation of diverse substrates ranging from ions to macromolecules across impermeable membranes.

ABC transporters function as either importers or exporters, but no example has been known yet of an ABC transporter that could function physiologically in both directions. ABC importers are found in prokaryotes, in which they mediate the uptake of nutrients, such as amino acids, oligopeptides, oligosaccharides and essential metals. ABC exporters are found in both prokaryotes and eukaryotes, in which they mediate efflux of toxins, drugs and lipids (Kos and Ford, 2009; Rees et al., 2009). Perhaps the most studied ABC transporter is human MDR1, which maintains cholesterol distribution across the PM but it also extrudes other lipophilic compounds, including chemotherapeutics agents, resulting in the multidrug resistance of tumor cells (Ambudkar et al., 2003; Higgins, 2007). Surprisingly, ABC proteins are involved not only in transport of substrate across membranes but also in non-transport-related processes such as translation of RNA and DNA repair (Davidson et al., 2008).

The common structure of ABC transporters consists of two transmembrane domains (TMDs) and two cytoplasmic nucleotide-binding domains (NBDs) which catalyze nucleotide hydrolysis. In eukaryotes these four domains are frequently fused creating single polypeptide but almost any configuration of these domains in membrane can be found. ABC proteins are localized to PM or in organelle membranes but always on the cytoplasmic side of the membrane (Rea, 2007; Kos and Ford, 2009; Locher et al., 2009; Rees et al., 2009) (for details see Fig. 4).

TMDs which provide substrate recognition and its translocation across the PM contain 12 hydrophobic α-helices (in eukaryotes typically 6 per domain). The membrane-spanning subunits are structurally heterogeneous and currently there are recognized three distinct sets of folds: type I ABC importer (ModABC transporter from *Archeoglobus fulgidus* (Hollenstein et al., 2007a, b), $MalFGK_2$ transporter from *Escherichia coli* (Oldham et al., 2007), ModB transporter from *Methanosarcina acetivorans* (Gerber et al., 2008), MetNI transporter from *Escherichia coli* (Kadaba et al., 2008)), type II ABC importer (BtuCD transporter from *Escherichia coli* (Locher et al., 2002), Hi1470 and Hi1471 transporters from *Haemophilus influenzae* (Pinket et al., 2007), BtuCDF transporter from *Escherichia coli* (Hvorup et al., 2007)) and ABC exporter folds (Sav1866 transporter from *Staphylococcus aureus* (Dawson and Locher, 2006, 2007), MsbA transporter from *Salmonella typhimurium, Escherichia coli, Vibrio cholerae* (Ward et al., 2007)).

The NBDs structures are strongly conserved and serve as energy source for substrate translocation using energy released upon ATP hydrolysis. The TMDs are coupled to NBDs via helices which dock into cavities on the interfacial surface of the NBDs (Hollenstein et al., 2007a,

b). The NBDs can be further divided into two constituent domains: a catalytic core domain, which contains the conserved P-loop or Walker A motif, Walker B motif, Q-loop and H-motif; and a more structurally diverse α-helical domain, which contains the ABC signature motif LSGGQ (Schmitt et al., 2003). The Q loop is presumed to be involved in the interaction of the NBD and TMD, particularly in the coupling of nucleotide hydrolysis to the conformational changes of the TMD during substrate translocation. The *H motif* or switch region contains a highly conserved histidine residue that is also important for the interaction of the ABC domain with ATP (Davidson and Chen, 2004; Davidson et al., 2008).

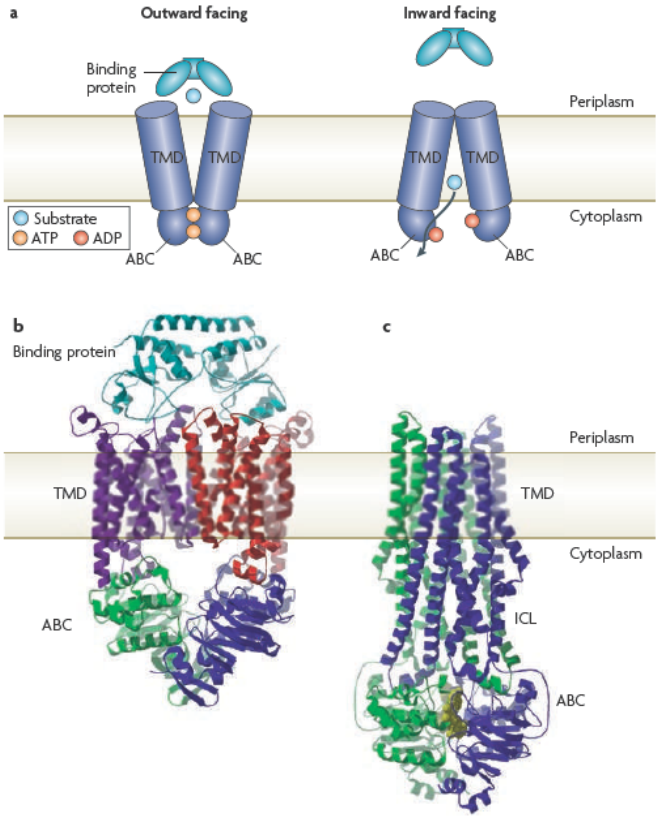


Figure 4. Molecular architecture of ABC transporters
a) Modular organization of ABC transporters, which are composed of two transmembrane domains (TMDs) and two ABC domains (or nucleotide-binding domains). The binding protein component that is required by importers is also shown. Two conformational states of the ABC transporter — outward facing and inward facing, with the substrate-binding site oriented towards the periplasmic (extracellular) and cytoplasmic (intracellular) regions, respectively — are depicted to show the alternating access mechanism of transport.
b) The *Escherichia coli* vitamin B12 importer BtuCDF22 consists of four subunits: the two TMD subunits (purple and red) and the two ABC subunits (green and blue). This complex also contains one copy the periplasmic binding protein (cyan).
c) The *Staphylococcus aureus* Sav1866 multidrug exporter consists of two subunits (green and dark blue), which contain a fused TMD and ABC domain. The nucleotides that are bound in this structure are shown by yellow space-filling models. ICL - intracellular loop, (adopted from Rees et al., 2009).

Export or import function seems to be determined by TMDs-NBDs interaction (Hollenstein et al., 2007a, b). Exporters have two intracellular loops from each TMD docked into NBDs in contrast to importers which are connected via one loop of each TMD.

The ABC dual transport activity, i.e. translocation of substrate in both directions in and out of the cell was not found out yet. However, Yang and Murphy (2009) indicated that ABCB4 expressed in *Schizosaccharomyces pombe* acts as an auxin importer under low substrate concentrations and as an auxin exporter under high substrate concentrations. Moreover, Yang and Murphy (2009) developed computational models of ABCB4 and ABCB19 based on the crystal structure of bacterial ABC transporter Sav1866 (Dawson and Locher, 2006). Structural comparisons indicate that ABCB4 and ABCB19 share a common architecture, but detailed sequence and structural analysis identified some differences. First, used COILS software program (http://www.ch.embnet.org/software/COILS_form.html) (Lupas et al., 1991) predicts

N-terminal coiled-coil structures in ABCB4 that are also found in the *Arabidopsis thaliana* ABCB14 guard cell malate importer (Lee et al., 2008), the *Coptis japonica* CjMDR1 putative berberine importer (Shitan et al., 2003), and ABCB21, a highly similar *Arabidopsis thaliana* ABCB protein (Verrier et al., 2008). No such N-terminal coiled-coil domain was found in the ABCB19 and ABCB1 exporters. Second, the hydrophobic region of TMH4 in ABCB4 is shifted below the membrane plane in the models and this fact could alter the TMD arrangement in ABCB4 and thus the direction of transport. Third, the linker domain of ABCB4, connecting NBD1 and TMD2, contains another coiled-coil structure, which is not found in the plant ABC importers CjMDR1, ABCB14 and ABCB21 or the ABCB1 and ABCB19 exporters, suggesting that this unique feature could also regulate ABCB4 activity. The switch between binding of the N-terminal coiled-coil domain to a binding protein or the linker coiled-coil domain could provide an explanation for the changeable directionality observed in ABCB4. Yang and Murphy (2009) also showed that IAA was primarily docked into two binding sites in the TMDs of ABCB19, while in ABCB4 the third unique binding site might function as a regulatory site, in which an interaction with IAA molecule activates export activity.

The high-resolution structures of eight intact ABC transporters (3 exporters and 5 importers) revealed different interactions between TMDs and NBDs and it seems that the substrate is probably one of the players responsible for conformational changes in the TMDs rather than passively transported molecule Kos and Ford (2009), and Rees et al. (2009).

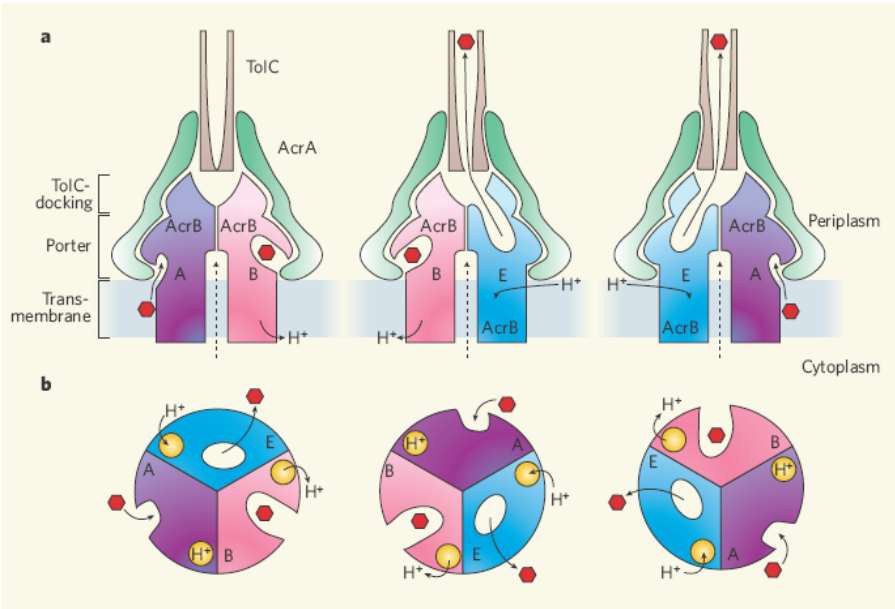
At present time, three-dimensional structures of four complete ABC exporters are available including crystal structures of MsbA from *Escherichia coli* in an open conformation (Chang and Roth, 2001), as well as its homologs from *Vibrio cholerae* in a closed form (Chang, 2003) and from *Salmonella typhimurium* complexed with lipopolysaccharide and ADP-vanadate (Reyes and Chang, 2005); furthermore, the crystal structure of Sav1866 from *Staphylococcus aureus* is available (Dawson and Locher, 2006).

The structures of ABC proteins gave a view on their mode of action. Murakami et al. (2006) and Seeger et al. (2006) reported the structure of AcrB from *Escherichia coli* (for details see Fig. 5), transporter that uses the energy of the transmembrane proton gradient, while Dawson and Locher (2006) detailed the structure of Sav1866 from S*taphylococcus aureus*, transporter that derives its power from the breakdown of ATP molecules.

Schuldiner (2006) reviewed that the transport mechanisms of these two model proteins have a common feature: and inward-facing conformation with the substrate-binding site accessible from the cell interior, and an outward-facing conformation with an extrusion pocket exposed to the external medium. The transition between the two conformations is energy dependent. An important question for ABC transporters concerns the ratio of ATP hydrolysed

per translocated substrate, and whether or not there is a complete coupling of these processes under physiological conditions.

Figure 5. The structure of the AcrB–drug complex and proposed mechanism of drug transport



a) The complex is seen from the side, with the drug shown as a red hexagon. The dotted line indicates a possible pathway for substrates moving from the cytoplasm. The complex contains three molecules of AcrB, AcrA accessory proteins and the TolC channel to the exterior. The drug is proposed to enter AcrB when it is in the access (A) conformation, before binding more closely to the porter domain of AcrB in the binding (B) conformation. It is then transported to the opposite face and is released from the extrusion (E) conformation of AcrB. Transport of the xenobiotic is powered by the proton (H^+) gradient across the membrane. **b)** The proposed ordered multidrug binding change mechanism of the three-unit AcrB complex (adopted from Schuldiner, 2006).

The enzymatic hydrolysis of ATP requires the presence of two sets of properly positioned groups: for binding the phosphates and for catalysing the attack of water on the γ-phosphate. For a large family of nucleotide-binding proteins, including ABC transporters, the conserved Walker A and Walker B motifs participate by binding the nucleotide phosphates and the Mg^{2+} that is coordinated to the nucleotide, respectively (reviewed by Rees et al., 2009).

The mechanism that characterizes the conformational changes associated with binding of substrate is the "alternating-access model". In this model, the substrate binding site alternates between outward- and inward-facing conformations. The relative binding affinities of these two conformations for the substrate largely determines the net direction of transport. For importers the outward-facing conformation will have higher binding affinity for substrate whereas the substrate binding affinity in exporters will be greater in the inward-facing conformation (Rees et al., 2009).

A model that describes the conformational changes in the NBD as a result of ATP binding and hydrolysis is the "ATP-switch model". This model presents two principal conformations of the NBDs: formation of a closed dimer upon binding two ATP molecules and dissociation to an open dimer facilitated by ATP hydrolysis and release of inorganic phosphate (P_i) and adenosine diphosphate (ADP). Switching between the open and closed dimer conformations induces conformational changes in the TMD resulting in substrate translocation (Higgins and Linton, 2004) (for details see Fig. 6).

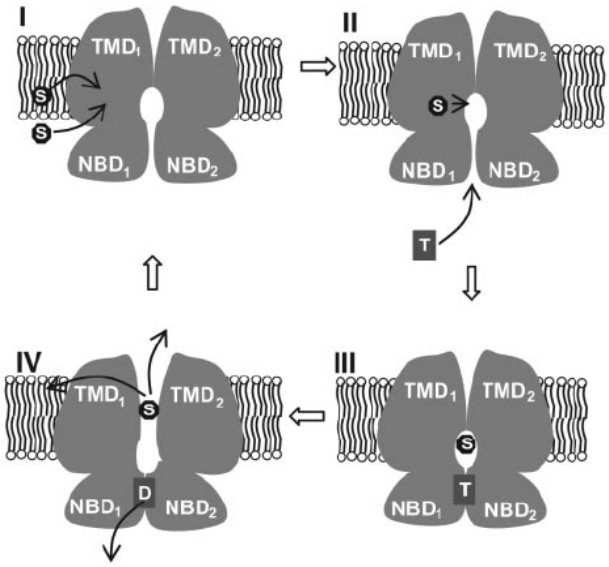


Figure 6. The figures schematize an ABC protein mediated transport
Substrate (S) coming from the cytosol or already within the membrane binds to a substrate binding site in one of the TMDs. This is followed by ATP (T) binding to the nucleotide-binding site localized at the interface between both NBDs. ATP binding and hydrolysis is accompanied by a structural rearrangement that allows the substrate to access to, and to be translocated through, a membrane path. Upon ADP (D) and phosphate dissociation, the protein returns to the initial state (adopted from Jasinsky et al., 2003).

However, general mechanism for the transport cycle of ABC transporters has not been fully elucidated but substantial structural and biochemical data has supported a model in which ATP binding and hydrolysis is coupled to transporter conformational changes.

4.6.3.2. Plant ABC proteins

The first complete inventory of the *Arabidopsis thaliana* ABC protein superfamily performed by Sánchez-Fernández et al. (2001b) revealed that the genome encodes 129 ABC proteins which were divided into 13 subfamilies (Fig. 7). Such a high number of plant ABC proteins is connected with their wide transport capacity together with exceptional metabolic versatility. More than 100 000 secondary metabolites have been identified in plants (*Arabidopsis* Genome Initiative, 2000), most of them would be toxic even in low concentrations, if they were not transported against steep concentration gradients across membranes out of the compartments in which they are synthesized (reviewed by Rea et al., 1998; Martinoia et al., 2000; Theodoulou, 2000; Yazaki, 2006). Later, Schulz and Kolukisaoglu (2006) brought new data from their phylogenomic analyses and Verrier et al. (2008) created new plant ABC proteins nomenclature.

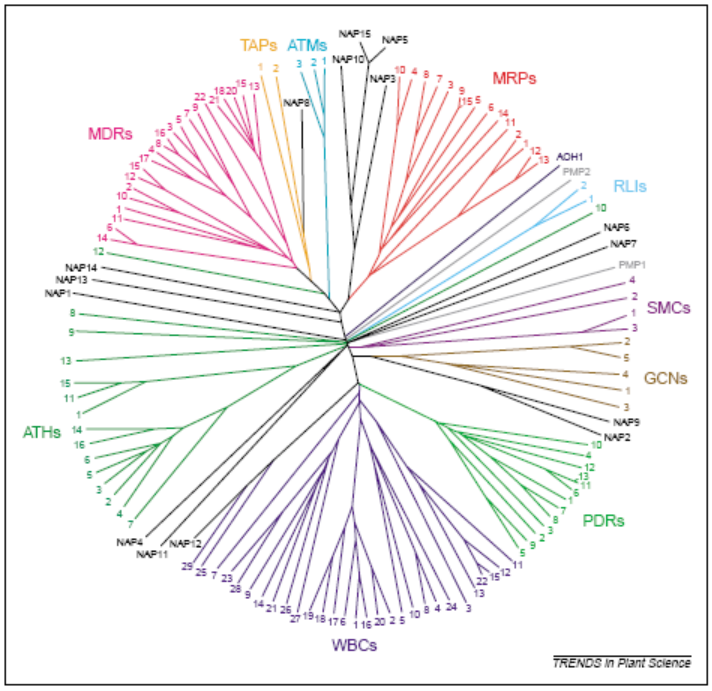


Figure 7. Phylogeny of *Arabidopsis* ABC proteins
The 13 subfamilies are shown in different colours. These are four subfamilies of full-molecule transporters (MDRs – multidrug resistance homologs, MRPs – multidrug resistance-associated protein homologs, PDRs – pleiotropic drug resistance homologs, AOH - ABC1 homolog), five subfamilies of half-molecule transporters (WBCs – white-brown complex homologs, ATHs – ABC2 homologs, ATMs – ABC transporter of the mitochondrion homologs, TAPs - transporter associated with antigen processing homologs and PMPs – peroxisomal membrane protein homologs) and four subfamilies of proteins lacking transmembrane spans (the RLIs – RNase L inhibitors homologs, GCNs – yeast GCN20, general control nonresponsible homologs, SMCs – structural maintenance of chromosomes homologs and NAPs – nonintrinsic ABC proteins) (adopted from Sánchez-Fernández R., 2001a).

Genome sequencing revealed 22 homologs to human and yeast MDR proteins in *Arabidopsis* (Sánchez-Fernández et al., 2001; Martinoia et al., 2002) and 17 homologs in rice (Jasinski et al., 2003), recently classified in ABCB protein subfamily (Verrier et al., 2008).

First plant homologue to mammalian *MDR* genes, the *ABCB1*, was cloned from *Arabidopsis thaliana* (Dudler and Hertig, 1992) with perspective to determine substrate specificity and find out its role in plant cells resistance to herbicides. The *ABCB1* homologue from barley (monocotyledonous plant) was isolated several years later (Davies, 1997). The first connection between ABCB1 and auxin transport was suggested by Sidler et al. (1998), when ABCB1 function in regulation of hypocotyl elongation has been proposed. Moreover, it was confirmed that the overexpression of ABCB1 protein causes resistance to herbicides (Windsor et al., 2003). Interestingly the compact stalk of agronomic important maize and sorghum mutants (*brachytic* (*br2*) and *dwarf* (*dw3*)) is caused by mutation in MDR-like gene (Multani et al., 2003). Later, Knöller et al. (2010) indicated that the function of ABCB1 in exporting auxin from meristematic regions is conserved in monocots and dicots, despite the pronounced differences observed in loss-of-function mutants (*br2* and *abcb1*).

Sidler et al. (1998) first demonstrated ABCB1 localization on PM. Later mainly apolar ABCBs cellular localization with partial apical localization in several tissues was proved (Geisler et al., 2005; Terasaka et al., 2005; Blakeslee et al., 2007; Cho et al., 2007).

Noh et al. (2001, 2003), Lin and Wang (2005) proved clear relation between auxin transport and ABCB1 or ABCB19 function, when they showed that *abcb1, abcb19* and double mutant as well exhibit pleiotropic growth phenotypes, like dwarf epinastic stature, curled leaves, and short stems and stamens. The *abcb19* and *abcb1abcb19* mutants also exhibit hypergravi- and hyperphototropic responses (Noh et al., 2003; Lin and Wang, 2005) and are defective in regulation of photomorphogenesis (Lin and Wang, 2005). Simultaneously high affinity binding of *Arabidopsis thaliana* ABCB1 and ABCB19 to the auxin efflux inhibitor NPA was confirmed (Noh et al., 2001; Murphy et al., 2002; Lin and Wang, 2005).

Polar auxin transport is reduced ~70% in *abcb1* and *abcb19*, while *pin1* exhibits a ~30% reduction (Blakeslee et al., 2007). However, *abcb* mutants show none of the defects in organogenesis that are seen in *pin1* (Noh et al., 2001). This suggests that ABCBs primarily regulate long-distance auxin transport and localized loading of auxin into the transport system and do not function in establishing the basal vectorial auxin flows that function in organogenesis (Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Bailly et al., 2008).

The ability of ABCB1 and ABCB19 to mediate auxin efflux in a heterologous system (yeast, mammalian HeLa and tobacco BY-2 cells) was already confirmed (Geisler et al., 2005; Petrášek et al., 2006). In root hair growth and NAA transport assays in transformed BY-2

tobacco cells, ABCB4 seemed to function as an auxin exporter (Cho et al., 2007). While these studies showed ABCBs efflux activity, interestingly in ABCB4-transgenic and mutant plants auxin uptake activity was also observed. Similarly to *ABCB1* and *ABCB19, ABCB4* expression is upregulated by exogenous auxin. Furthermore, the *ABCB4* expression increases sensitivity of yeast for auxin and its analogues (Santelia et al., 2005). ABCB4 is strongly expressed in roots and regulates auxin distribution in lateral root cap thus affecting root gravitropism (Santelia et al., 2005, Terasaka et al., 2005). As it was already mentioned, ABCB4 exhibits structural similarity to the berberine uptake transporter CjMDR1/CjABCB1 from the medicinal plant *Coptis japonica* (Shitan et al., 2003) and, to a lesser extent, to the ABCB14 malate uptake transporter from *Arabidopsis thaliana* guard cells (Lee et al., 2008). The role of ABCB proteins (1, 4 and 19) in plant development and in cellular or long-distance auxin transport was reviewed by Geisler and Murphy (2006) and Rea (2007).

The enhancement of *ABCB19* expression in endodermal and pericycle cells which form the border of auxin streams suggested the inhibitory role of ABCB19 in auxin lateral movement. This was tested by measuring auxin "escape" from stellar stream by specially designed auxin transport assays (Bandyopadhyay et al., 2007; Blakeslee et al., 2007). Lewis et al. (2007) used *abcb1* and *abcb4* mutants to dissect the roles of two antiparallel auxin streams (acropetal and basipetal) in root growth and development and they confirmed importance of ABCB1 and ABCB4 transporters for root auxin transport, while they showed that mutations in *ABCB1* reduced acropetal auxin transport in roots by 80%, without affecting basipetal transport, and conversely, mutations in *ABCB4* blocked 50% of basipetal transport without affecting acropetal transport. (Wu et al., 2007) used the same mutants to investigate the role of the auxin transport streams in lateral root growth development.

A breakthrough into way of ABCBs action in auxin transport was revealing the role of ABCB1 and ABCB19 in PIN1 polar distribution in PM (Noh et al., 2003; Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Titapiwatanakun et al., 2009; Titapiwatanakun and Murphy, 2009). When PINs and ABCBs are co-expressed in heterologous systems, their interaction enhances the rate of auxin efflux and increases the substrate specificity and efflux inhibitor sensitivity (Blakeslee et al., 2007). Moreover, the loss of auxin transport specificity was observed in *pin1* and *abcb19* mutants, which confirms the importance of coordinated ABCB-PIN action. On the other hand, the interaction between auxin influx carrier AUX1 and ABCB1 or ABCB19 was not observed. ABCBs and PINs exhibit overlapping expression patterns and co-localize in PIN polar domain (Blakeslee et al., 2007), but the mechanism of vesicular PIN1 trafficking is not involved in ABCB19 regulation (Titapiwatanakun et al., 2009; Titapiwatanakun and Murphy, 2009). Even if possible role of ABCB in PIN targeting was proposed (Noh et al., 2003), further analyses

showed that ABCB probably only stabilizes PINs in detergent resistant microdomains (Titapiwatanakun et al., 2009; Titapiwatanakun and Murphy, 2009) pointing at the composition of lipid membrane as an important factor in regulation of integral membrane proteins such as PINs and ABCBs. It was found that ABCB19 as well as PIN1 proteins are localized in sterol-rich detergent-resistant membrane fractions. In mutant *abcb19* PIN1 protein was not detected probably due to the loss of ABCB19-PIN1 interaction. The reason why PIN1 transport activity is not detected in *Saccharomyces cerevisiae* was probably a lack of such sterol-rich microdomains (Titapiwatanakun et al., 2009).

Relative to ABC proteins transport plasticity researchers were and are looking for specific ABC inhibitors to effectively block their function. NPA was initially used in affinity chromatography to isolate the ABCB1, 4, and 19 proteins (Murphy et al., 2002; Geisler et al., 2003; Terasaka et al., 2005). Murphy et al. (2002) also identified high and low affinity NPA binding sites in *Arabidopsis thaliana* membranes, consecutively FKBP immunophilin-like protein, TWD1/FKBP42 was co-purified with the ABCBs and it has been proposed to induce conformational changes in ABCB1 and ABCB19 (Geisler et al., 2003; Bouchard et al., 2006; Bailly et al., 2008) (for details about TWD1 see chapter 4.6.3.3.). Other inhibitor gravacin was originally identified as an inhibitor of gravitropic bending in hypocotyls (Surpin et al., 2005). Wild-type *Arabidopsis thaliana* seedlings treated with gravacin resulted in reductions of auxin transport that were similar to those seen in *abcb19*, while gravacin treatment of *abcb19* mutants resulted in further reductions in auxin transport (Rojas-Pierce et al., 2007). Treatment with gravacin did not alter the gravitropic response of *twd1*, and microsomes derived from *twd1* showed reduced binding to gravacin (Rojas-Pierce et al., 2007; Bailly et al., 2008). However, the immunolocalization of ABCB19 was unchanged in *twd1* mutants compared to wild type (Titapiwatanakun et al., 2009), suggesting that FKBP42 is more likely to function in activation, rather than localization of ABCB1/19 to the PM. Kim et al. (2010) identified BUM as another specific inhibitor of ABCB-related auxin transport.

Compared with the more dynamic processes regulating membrane localization of the AUX1 and PIN-family proteins, ABCB19 is more stably situated on the PM (Titapiwatanakun et al., 2009). Whereas the dynamic cycling of PIN1 is disrupted by short-term treatments with actin depolymerising compound latrunculin B (Geldner et al., 2001), the localization of ABCB19 is unaffected. Similarly ABCB19 subcellular localization is insensitive to short-term treatments with the microtubule depolymerizing compound oryzalin and is also insensitive to wortmannin (Titapiwatanakun et al., 2009). However, treatment with gravacin does interfere with the trafficking of ABCB19 to the PM resulting in aggregation of some ABCB19 protein in an unidentified compartment that does not co-localize with the endocytic marker FM4-64 (Rojas-

Pierce et al., 2007). As the trafficking of ABCB19 to the PM is not BFA-sensitive, it is not mediated by GNOM-dependent mechanisms (Titapiwatanakun et al., 2009). However, ABCB19 appears to be trafficked by GNOM-LIKE1 (GNL1), a BFA-insensitive ARF-GEF in the GNOM family that mediates vesicular ER-Golgi trafficking (Richter et al., 2007; Teh and Moore, 2007). In *Arabidopsis thaliana*, mutations in GNL1 exhibit a reduction in the abundance and PM localization of ABCB19, but not of PIN1 and PIN2. On the other hand, ABCB1 does aggregate in intracellular bodies with PIN2 after BFA treatment, suggesting that it is less stable and more readily endocytosed than ABCB19 (Blakeslee et al., 2007; Titapiwatanakun et al., 2009). This is even more likely, as ABCB1 exhibits stronger interactions with both FKBP42/TWD1 and the auxin transport inhibitor NPA (Murphy et al., 2002; Geisler et al., 2003; Bouchard et al., 2006; Bailly et al., 2008).

4.6.3.3. ABCB proteins are functionally regulated via immunophilin TWISTED DWARF 1

FK506-binding proteins (FKBPs), together with unrelated cyclophilins, belong to the immunophilins, an ancient and ubiquitous protein family (Schreiber, 1991; Harrar et al., 2001; He et al., 2004; Romano et al., 2004a, b). They were first described as receptors for immunosuppressive drugs in animal and human cells, FK506 and cyclosporine A, respectively (Schreiber, 1991). All FKBP-type immunophilins share a characteristic peptidyl-prolyl cis-trans isomerase domain (PPIase domain or FK506-binding domain (FKBD)) making protein folding a key feature among immunophilins (Schiene-Fischer and Yu, 2001; Bailly et al., 2006).

Immunophilins were shown to be distributed throughout the plant cell (Breiman et al., 1992; Luan et al., 1994), and it has been recently confirmed by detailed subcellular localization (e.g. Geisler et al., 2003; Bouchard et al., 2006). Twenty-three FKBP-type proteins have been identified in the *Arabidopsis thaliana* genome; however, their individual function is poorly understood. Anyway, the discovery of plant immunophilins has not only demonstrated conservation of these proteins in a full spectrum of biological systems but has also provided clues to their potential functions (Romano et al., 2004a, b). For example, some plant cyclophilin genes have been shown to be induced by a variety of biotic and abiotic stresses, suggesting that they may play a role in environmental response processes (Chou and Gasser, 1997; Kurek et al., 1999).

The FKBP domain of FKBP42 has been demonstrated to physically interact with the C-terminal NBDs of PM-localized transporters ABCB1 and ABCB19 (Geisler et al., 2003), whereas the TPR domain appears to be responsible for functional association with vacuolar transporters ABCC1 and ABCC2 (Geisler et al., 2004). The *Arabidopsis thaliana* mutant *twisted dwarf1* (*twd1)* Perez-Perez et al., 2004) lacks FKBP42 and displays a drastic pleiotropic auxin-

related phenotype that includes reduced development and cell elongation (dwarfism), and disoriented growth of all organs both on the epidermal and whole plant level (Geisler et al., 2003). Originally, Kamphausen et al. (2002) and Bouchard et al. (2006) predicted TWD1 to be membrane-localized (PM and tonoplast), but newly, Wu et al. (2010) showed TWD1 localization in ER, moreover they proved that mutations in *TWD1* caused mislocalization of ABCB1, ABCB4, and ABCB19 to the ER instead of PM comparing to unrelated PIN2 PM localization.

Earlier, ABCB1 and ABCB19 have been shown to directly mediate cellular auxin efflux (Geisler et al., 2005; Lin and Wang, 2005). Strikingly, the double mutant *abcb1/abcb19*, but not the corresponding single mutants, shares with *twd1* highly similar auxin-related phenotypes: dwarfism and agravitropic roots (Bailly et al., 2006; Bouchard et al., 2006). Bouchard et al. (2006) proved that cellular efflux of IAA from mutant cells is reduced compared with that from wild-type cells in the order wild type > *abcb1* > *abcb19* >> *abcb1/abcb19* ≥ *twd1*. Furthermore the overexpression of TWD1 has no effect on IAA export, whereas up-regulation of the ABC transporters ABCB1 and ABCB19 strongly enhances efflux. Expression and localization of PIN1 and PIN2 are not altered in *twd1*.

The simplest mechanistic model proposed TWD1-induced conformational changes in the C- termini of ABCB1 and ABCB19 increase ATP access to the second ATP-binding site of these proteins. In the absence of TWD1, the ATP-binding site would be blocked, leaving ABCB1/ABCB19 in an inactive state (Bouchard et al., 2006).

Recently, Bailly et al. (2008) reported specific disruption of ABCB-TWD1 interaction by NPA and flavonoids using BRET technique. This is in line with previous findings demonstrating NPA binding to plant ABCBs and inhibition of ABCB-mediated auxin transport by NPA (Santelia et al., 2005; Terasaka et al., 2005; Bouchard et al., 2006). In contrast to NPA, the auxin transport inhibitors (ATIs) 2,3,5-triiodobenzoic acid (TIBA) and 2-carboxylphenyl-3-phenylpropan-1,3-dione (CPD) had no significant effect on the ABCB1-TWD1 complex stability and auxin transport (Bailly et al., 2008). This is not unexpected, as TIBA is structurally unrelated and has been shown to displace NPA binding only partially and even to own weak auxin activity, suggesting a different locus and mode of action compared with NPA (Petrášek et al., 2003). Conversely, the flavonol quercetin was the most capable of disrupting ABCB1-TWD1 interaction (Bailly et al., 2008) probably due to the highest efficiency of quercetin in competing with NPA for auxin transporter binding sites (Jacobs and Rubery, 1988).

It seems reasonable to assume that impaired cell elongation and disoriented growth of *twd1* plants result from non-sufficient auxin transport and above all it supports the idea of TWD1 central position in ABCB-mediated auxin transport.

4.6.3.4. Regulation of ABCB proteins by flavonoids

Flavonoids are plant secondary polyphenolic metabolites and serve as important nutraceuticals. They have health-promoting effects, including antioxidant, anticarcinogenic, antiviral, and anti-inflammatory activities; however, the cellular targets of the *in vivo* protein remain largely unknown (Taylor and Grotewold, 2005; Morris and Zhang, 2006), apart from interactions of flavonoids with animal ABC transporters involved in drug absorption, distribution, excretion and resistance. Until now many studies have demonstrated inhibition of animal ABC transporters, mainly ABCB1, ABCC1, ABCC2 and ABCG2 by flavonoids (Morris and Zhang, 2006; Alvarez et al., 2010).

Flavonoids are widely distributed throughout the plant kingdom and are abundant in many flowers, fruits and leaves (reviewed by Winkel-Shirley, 2002; Taylor and Grotewold, 2005; Zhao and Dixon, 2009; Alvarez et al., 2010; Buer et al., 2010). In plants, besides playing a role in defence responses to environmental impacts (Treutter, 2005), allelopathy (Bais et al., 2006) or root nodule development (Subramanian et al., 2007; Mathesius, 2008) and modulating the levels of reactive oxygen species (ROS) (Taylor and Grotewold, 2005; Bais et al., 2006), they have been also shown to inhibit polar auxin transport (PAT) and consequently to enhance localized auxin accumulation (Stenlid, 1976; Marigo and Boudet, 1977; Jacobs and Rubery, 1988; Murphy et al., 2000; Brown et al., 2001; Peer et al., 2001, 2004, 2007). The regulatory impact of flavonoids on PAT was initially based on their ability to compete with NPA or herbicide for transporter binding sites. This concept is further supported by auxin-related phenotypes of *Arabidopsis thaliana* transparent testa mutants with altered flavonoids levels (Murphy et al., 2000; Brown et al., 2001; Peer et al., 2001, 2004; Taylor and Grotewold, 2005). At the present time flavonoids are considered to be transport regulators or modulators (Peer et al., 2007); nevertheless, the mechanisms by which flavonoids interfere with auxin efflux components are not yet clear.

It has been shown that treatment with flavonoids and mammalian MDR/PGP inhibitors reverse efflux of human MDR substrates to the point of net retention in mammalian cells (Zhang and Morris, 2003; Morris and Zhang, 2006). Consecutively it was confirmed that flavonoids are also functioning as inhibitors of plant ABCBs (Geisler et al., 2005; Terasaka et al., 2005; Bouchard et al., 2006), probably by mimicking ATP and competing for ABCB NBDs.

In addition to regulating ABCBs, a lack of flavonoids in *Arabidopsis thaliana* altered the expression and localisation of certain PIN proteins, and it was suggested that flavonoids could act by targeting PIN intracellular cycling, at least in the root tip. However, it is likely that PIN protein localisation is not regulated by flavonoids directly but by auxin localisation itself in a positive feedback loop (Peer et al., 2004). This could be regulated at the level of vesicle cycling

as auxin was shown to inhibit internalisation of PIN proteins mediated by BIG (calossin-like protein required for PAT), thus auxin could increase its own efflux from cells (Paciorek et al, 2005).

Studies in flavonoid-deficient *Arabidopsis thaliana* mutants confirmed that these plants had higher rates of auxin transport whereas mutants over accumulating flavonols show decreased auxin transport rates (Murphy et al., 2000; Brown et al, 2001; Peer et al., 2004). Flavonoids could be an ideal link between auxin transport and the environment because they are accumulated in response to a variety of environmental stimuli (Buer and Muday et al., 2004; Taylor and Grotewold, 2005). The co-localisation of flavonoids at sites of high auxin concentration supports their role in auxin transport control (Murphy et al., 2000; Peer et al., 2001; Buer and Muday, 2004; Buer et al., 2006).

Although we have lot of new information about flavonoids action in plants, there are still some questions connected with flavonoids activity e.g. what is their effect on AUX1 and LAX carriers or how much of free flavonoids remain in the cytoplasm to modulate the trafficking of the activity of auxin transporters?

Up to now, there is a lot of information about various auxin transporters and their role in plant development. However, there are also remaining open questions, many of them related to ABCB transporters and their mechanism of action in auxin transport.

5. Materials and Methods

5.1. Plant material

Cells of tobacco line BY-2 (*Nicotiana tabacum* L., cv. Bright-Yellow 2) (Nagata et al., 1992) were cultured in liquid medium (3% [w/v] sucrose, 4.3 $g.l^{-1}$ Murashige and Skoog salts, 100 $mg.l^{-1}$ inositol, 1 $mg.l^{-1}$ thiamin, 0.2 $mg.l^{-1}$ 2,4-D, and 200 $mg.l^{-1}$ KH_2PO_4 [pH 5.8]) in darkness at 26°C on an orbital incubator (Sanyo Gallenkamp, Schöeller Instruments Inc., Prague, Czech Republic; 150 rpm, 32 mm orbit) and subcultured weekly. Stock BY-2 calli were maintained on media solidified with 0.6% (w/v) agar and subcultured monthly. BY-2 cells were stably transformed by co-cultivation with *Agrobacterium tumefaciens* strain GV2260 carrying gene constructs with *Arabidopsis thaliana* genes under various promoters with various tags: pGVG::ABCB1-*myc* and pGVG::ABCB19-HA (Blakeslee et al., 2007), ABCB4::ABCB4:GFP (Cho et al., 2007), PIN1::PIN1:GFP (Benková et al., 2003) and pGVG::PIN7:GFP (Petrášek et al., 2006), as described in An et al. (1985) and Petrášek et al. (2003). The pGVG-ABCB1-*myc* and pGVG-ABCB19-HA plasmids were constructed by cloning the whole genomic coding region of *ABCB1* and *ABCB19* genes fused with the respective tag by primer extension PCR to pTA7002 (Aoyama and Chua, 1997), and transformed to the *UAS::GUS* (Weijers et al., 2003) line. Fifteen independent transgenic lines were analysed for each construct. Transgenic BY-2 cells and calli were maintained on the media supplemented with 40 $\mu g.ml^{-1}$ hygromycin or kanamycin and 100 µg.ml-1 cefotaxim. Expression of PIN and ABCB genes in tobacco cells was induced by the addition of dexamethasone or estradiol (DEX or EST, 1 µM, 24 hours, except for stated otherwise) at the beginning of the subcultivation period.

5.2. Expression and localization analysis

Tobacco RNA was isolated using the Plant RNA Qiagen Mini-Prep and RT-PCR performed using Qiagen® OneStep RT-PCR kits according to the manufacturer's protocols.

For Western Blots total protein fraction from GVG-ABCB19 and GVG-ABCB1 tobacco BY-2 cells was obtained after homogenization in liquid nitrogen. The frozen powder was then mixed with an equal volume of extraction buffer (50 mM Tris-HCl, pH 6.8; 2% (w/v) SDS; 36% (w/v) urea; 30% (w/v) glycerol; 5% (v/v) mercaptoethanol; 0.5 % (w/v) Bromphenol Blue), vortexed for 1 min, boiled for 3 min, and centrifuged at 13.000 rpm and 4°C for 5 min. The supernatant was transferred into a new tube and re-centrifuged at 13.000 rpm and 4°C for 5 min. The resulting supernatant was defined as total protein extract and stored at –20°C until use.

For immunolocalization GVG-ABCB19-HA and GVG-ABCB1-myc tobacco BY-2 cells were pre-fixed 30 min in 100 µM MBS and 30 min in 3.7% (w/v) PFA buffer consisting of 50

mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, (pH 6.9), at 25°C and subsequently in 3.7% (w/v) PFA and 1% Triton X-100 (w/v) in stabilizing buffer for 20 min. After treatment with an enzyme solution (1% (w/v) macerozyme and 0.2% (w/v) pectinase) for 7 min at 25°C and 20 min in ice cold methanol (at -20°C), the cells were attached to poly-L-lysine coated coverslips and treated with 1% (w/v) Triton X-100 in microtubule stabilizing buffer for 20 min. Then the cells were treated with 0.5% (w/v) bovine serum albumin in PBS and incubated with primary antibody for 45 min at 25°C. After washing with PBS, a secondary antibody in PBS was applied for 1 h at 25°C. Coverslips with cells were carefully washed in PBS, rinsed with water with Hoechst 33258 (0.1μg/ml) and embedded in Mowiol (Polysciences) solution. The following antibodies and dilutions were used: anti-HA (Sigma-Aldrich; 1:500), anti-*myc* (Sigma-Aldrich; 1:500), TRITC-(Sigma-Aldrich; 1:200), FITC-(Sigma-Aldrich; 1:200). Slides were observed using a microscope Nikon Eclipse E600 equipped with appropriate filter sets, DIC optics and color digital camera (DVC 1310C, USA).

5.3. Chemicals

Unless stated otherwise, all chemicals were supplied by Sigma-Aldrich, Inc. (St. Louis, USA). 1-naphthylphthalamic acid (NPA), was supplied by OlChemIm Ltd. (Olomouc, Czech Republic). ^{3}H-benzoic acid (BeA [4^{3H}]), ^{3}H-indole-3-acetic acid (IAA) and ^{3}H-2,4-dichlorophenoxyacetic acid (2,4-D) (all of specific radioactivity 20 Ci.mmol^{-1}) and ^{3}H-naphthalene-1-acetic acid (NAA) (specific radioactivity 25 Ci.mmol^{-1}) were supplied by American Radiolabeled Chemicals, Inc. (St. Louis, USA).

5.4. BY-2 cell microscopy

Cell densities were determined by counting cells in at least 8 aliquots of each sample using Fuchs-Rosenthal haemocytometer slide and inverted microscope Zeiss Axiovert 40C. Phenotype analyses (cell lengths and diameters) were observed with microscope Nicon Eclipse E600 (Japan) and images were grabbed with colour digital camera (DVC 1310C, USA) using LUCIA image analysis software (Laboratory Imaging, Prague, Czech Republic). For all in vivo observations Zeiss LSM510-DUO confocal microscope with a 40x C-Apochromat objective (NA=1.2W) was used.

5.5. Auxin accumulation assays in BY-2 cells

Auxin accumulation by two-day old cells was measured according to the Delbarre et al. (1996) as modified by Petrášek et al. (2006). Treatments were repeated at least two times and averaged values (± standard errors) were expressed as pmols of particular auxin accumulated per 10^6 cells. Depending on experiment, 10 μM 2-NOA or NPA (both from 50 mM DMSO stock) or

cold auxins 5 μM IAA and 2,4-D (50 mM ethanol stocks) were added at the beginning of the accumulation assay (together with the addition of radioactively labelled auxin) or 30 min before beginning of the accumulation assay (to increase inhibitor effect). The ^{3}H-2,4-D, ^{3}H-BeA, ^{3}H-IAA and ^{3}H-NAA were added to the cell suspension to give a final concentration of 2 nM. After a timed uptake period, 0.5-ml aliquots of cell suspension were collected and accumulation of label was terminated by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. The cell cakes and filters were transferred to scintillation vials, extracted in 0.5 ml of 96% ethanol for 30 min, and afterwards 4 ml of scintillation solution (EcoLite Liquid Scintilation Fluid, MP Biomedicals, Solon, USA) were added. Radioactivity was determined by liquid scintillation counter Packard Tri-Carb 2900TR (Packard-Canberra, Meridian, CT, USA) with automatic correction for quenching.

5.6. HPLC metabolic profiling

Two days old BY-2 cells were prepared for the experiment by equilibration in uptake buffer as for accumulation assays described (Petrášek et al., 2006). Experiments were done in uptake buffer and under standard cultivation conditions. Cells were incubated with addition of 20 nM ^{3}H-BeA, ^{3}H-IAA, ^{3}H-NAA and ^{3}H-2,4-D for a period of 0 and 10 min. Cells and media (uptake buffer) were collected and frozen (200 mg of fresh weight and 10 ml per sample). Extraction and purification of auxin metabolites in cells and media were performed as described (Dobrev and Kamínek, 2002, Dobrev et al., 2005). The radioactive metabolites were separated on HPLC. For this method column LunaC18 (2), 150 × 4.6 mm, 3 μm (Phenomenex, Torrance, CA, USA) was used, mobile phase A was 40 mM CH_3COONH_4, (pH 4.0) and mobile phase B was CH_3CN/CH_3OH, 1/1, (v/v). Flow rate was 0.6 ml min^{-1} with linear gradient 30–50 % B for 10 min, 50–100 % B for 1 min, 100 % B for 2 min, 10–30 % B for 1 min. The column eluate was monitored by a Ramona 2000 flow-through radioactivity detector (Raytest GmbH, Straubenhardt, Germany) after online mixing with three volumes (1.8 $ml.min^{-1}$) of liquid scintillation cocktail (Flo-Scint III, Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). The radioactive metabolites were identified on the basis of comparison of their retention times with authentic standards. For the results presentation the total integrated area of chromatogram plots was normalized based on the equalization of total accumulated radiolabel.

6. Results

6.1. Chapter 1 - PIN proteins perform a rate-limiting function in cellular auxin efflux

Jan Petrášek, Jozef Mravec, Rodoplhe Bouchard, Joshua J. Blakeslee, Melinda Abas, Daniela Seifertová, Justyna Wiśniewska, Zerihun Tadele, **Martin Kubeš**, Milada Čovanová, Pankaj Dhonukshe, Petr Skůpa, Eva Benková, Lucie Perry, Pavel Křeček, Ok Ran Lee, Gerald R. Fink, Markus Geisler, Angus S. Murphy, Christian Luschnig, Eva Zažímalová, Jiří Friml

Science 312, 914-918, 2006

This article is based on collaboration between Dr. Eva Zažímalová laboratory and several foreign laboratories. Here we have showed the specific and rate-limiting function of PIN proteins in cellular auxin efflux.

My contribution to this article was the transformation of BY-2 cells with GVG-ABCB19-HA construct, characterization of cell lines including expression analyses (RT-PCR, immunofluorescence localization and western blot) and auxin accumulation assays with radiolabeled auxins (see Fig. 4A, B, C below). My main finding is that the ABCB19-mediated NAA efflux was notably less sensitive to NPA compared to the PIN7-mediated auxin efflux.

Our part of this paper was supported by the Grant Agency of the Academy of Sciences of the Czech Republic, project A6038303 and the Ministry of Education of the Czech Republic, projectLC06034.

6.2. Chapter 2 - Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development

Jozef Mravec, **Martin Kubeš**, Agnieszka Bielach, Vassilena Gaykova, Jan Petrášek, Petr Skůpa, Suresh Chand, Eva Benková, Eva Zažímalová and Jiří Friml

Development 135, 3345-3354, 2008

This paper is based on collaboration between Dr. Jiří Friml and Dr. Eva Zažímalová laboratories. Here, we have analyzed the importance of the functional interaction between PIN- and ABCB/PGP-dependent auxin transport in plant development.

My contribution to this article was in the characterization of PIN- and ABCB/PGP-mediated auxin efflux at the cellular level. For this comparison I used DEX-inducible BY-2 cell lines GVG-ABCB19-HA and GVG-PIN7.

To find out how increased auxin efflux influences cellular behaviour of BY-2 cells, I examined the growth and morphology of DEX-treated and untreated cells in these two cell lines. After induction of PIN7 or ABCB19-HA expression, identical phenotypical changes occurred: cells ceased to divide, started to elongate, and formed and accumulated starch granules. Importantly, this cellular behaviour could be mimicked by cultivation of cells in medium with a decreased amount of auxin or no auxin (see Fig. S1E -G in the supplementary material). All phenotypic changes induced by overexpression of PIN7 in the GVG-PIN7 line were completely reversed by application of the auxin efflux inhibitor NPA (Fig. 1C, G; see Fig. S1J, K in the supplementary material). By contrast, after induction of ABCB19-HA expression in the GVG-ABCB19-HA line, NPA treatment was ineffective in rescuing auxin starvation phenotypes (Fig. 1F, G; see Fig. S1J, K in the supplementary material).

Our part of this paper was supported by the Ministry of Education, Youth and Sports of the Czech Republic, project LC06034 and by the Grant Agency of the Academy of Sciences of the Czech Republic (KJB600380604).

6.3. Chapter 3 - Probing plant membranes with FM dyes: tracking, dragging or blocking?

Adriana Jelínková, Kateřina Malínská, Sibu Simon, Jürgen Kleine-Vehn, Markéta Pařezová, Přemysl Pejchar, **Martin Kubeš**, Jan Martinec, Jiří Friml, Eva Zažímalová, Jan Petrášek

The Plant Journal 61, 883-892, 2010

This article is based on collaboration between Dr. Eva Zažímalová, Dr. Jiří Friml and Dr. Jan Martinec laboratories. The goal of this manuscript is to describe side-effects of the FM (Fei Mao) styryl dyes, which are widely used probes that label processes of endocytosis and vesicle trafficking in eukaryotic cells.

My contribution to this article was the transformation of BY-2 cells with ABCB4::ABCB4:GFP construct, characterization of cell line including expression analyses (immunofluorescence localization) and analysis of cell phenotype. I was involved in microscopy analysis of FM 4-64 effect on ABCB4-GFP plasma membrane localization in BY-2 cells expressing ABCB4-GFP.

FM styryl dyes were used for characterization of endosomal trafficking of proteins involved in the auxin efflux machinery in BY-2 cell lines. Direct application of 2 μM FM 4-64 to BY-2 cells constitutively expressing either PIN1–GFP or ABCB4–GFP resulted in transient internalization of these proteins from the PM. Control cells showed localization of PIN1–GFP and ABCB4–GFP in the PM and the cortical cytoplasm (Figure 2a, b, 0 min, and Figure S1a). Prolonged incubation with FM 4-64 (up to 30 min) resulted in remarkable endocytosis of FM 4-64, but PIN1–GFP as well as ABCB4–GFP vesicles disappeared from the cortical cytoplasm, restoring a control-like situation (i.e. localization on the PM; Figure 2a, b, 30 min).

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6.4. Chapter 4 - Auxin influx inhibitors 1-NOA, 2-NOA, and CHPAA interfere with membrane dynamics in tobacco cells

Martina Laňková, Richard Smith, Bedřich Pešek, **Martin Kubeš**, Eva Zažímalová, Jan Petrášek, Klára Hoyerová

Journal of Experimental Botany 61, 3589-3598

This paper is the result of Dr. Eva Zažímalová laboratory with a small contribution from the Univ. Bern, Switzerland. In this report, we reveal the mechanism of action of the auxin influx inhibitors 1-NOA, 2-NOA, and CHPAA by direct measurements of auxin accumulation, cellular phenotypic analysis, as well as by localization studies of *Arabidopsis* auxin carriers heterologously expressed in BY-2 cells.

My contribution to this article was in the transformation of BY-2 cells with ABCB4::ABCB4:GFP construct, characterization of cell line including expression analyses (immunofluorescence localization) and analysis of cell phenotype. I was involved in microscopy analysis of the effects of the auxin influx inhibitors (1-NOA, 2-NOA and CHPAA) on subcellular distribution of ABCB4-GFP fusion proteins in tobacco BY-2 cells.

To determine whether the auxin influx inhibitors (1-NOA, 2-NOA and CHPAA) can influence AUX1, PIN1, ABCB4 proteins distribution, *XVE::EYFP:AUX1*, *XVE::PIN1:GFP, ABCB4::ABCB4:GFP* BY-2 cell lines were used. In control situation the PM fluorescence of all fusion proteins was observed without visible vesicles in the cortical cytoplasm (Fig. 4A, E, I). After 3 h treatment with 20 μM 1-NOA, both PIN1:GFP and EYFP:AUX1 (Fig. 4B, F) were observed in vesicles reminiscent of endosomes and located in the cortical cytoplasm. Similar treatment with 2-NOA induced these patches as well, but the effect was weaker (Fig. 4C, G). A 3 h exposure to 20 μM CHPAA did not induce any protein redistributions (Fig. 4D, H). Interestingly, only after 24 h treatment with 1-NOA ABCB4:GFP fusion protein appeared in a form of few membrane aggregates (Fig. 4J, K, L).

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6.5. Chapter 5 - Arabidopsis ABCB4 is a substrate-activated homeostatic regulator of auxin levels in Arabidopsis roots

Martin Kubeš*, Haibing Yang*, Gregory L. Richter, Yan Cheng, Xia Wang, Ewa Młodzińska, Nicola Carraro, Jan Petrášek, Eva Zažímalová, Klára Hoyerová, Wendy Ann Peer, Angus S. Murphy

* These authors contributed equally to this work.

The Plant Journal, *resubmitted*

This article is based on collaboration between Dr. Eva Zažímalová and Dr. Angus S. Murphy/Wendy A. Peer laboratories. The main message of this paper is the suggestion of mechanism, where ABCB4 gradually changes its auxin uptake to auxin efflux activity with increasing auxin levels. Moreover, we confirm that 2,4-D is transported by ABCB4 not only into the cells but also partially out from the cells and non-competitively interacts with ABCB4 to block its auxin efflux activity. This inhibition might be responsible for the effectiveness of 2,4-D as a commercial herbicide.

My contribution to this article was in the transformation of BY-2 cells with ABCB4::ABCB4:GFP construct, characterization of this cell line including expression analyses (immunofluorescence localization), analysis of cell phenotype, determination of cell growth curve, accumulation assays using radiolabeled (^{3}H-2,4-D, ^{3}H-BeA, ^{3}H-IAA and ^{3}H-NAA) and cold (2,4-D, IAA, NAA) auxins, and auxin transport inhibitors (2-NOA, NPA).

Expression of ABCB4-GFP in BY-2 cells produces phenotypes consistent with auxin efflux (Fig. 4A), but no cell elongation like in ABCB19 or PIN1 cells was observed (Fig. 4B). PM localisation of ABCB4-GFP is mainly non-polar compared to PIN1-GFP (Fig. 4C). Decrease in transversal localisation may be a consequence of faster lateral diffusion or trafficking of ABCB4 compared to PIN1-GFP as indicated by FRAP analysis (Fig. 4D). ABCB4-overexpressing cells exhibit increased ^{3}H-NAA net uptake after NPA treatment (Fig. 5A), suggesting that ABCB4 has also NPA-sensitive efflux activity. Further, these cells showed only a slight decrease in net ^{3}H-BeA retention (Fig. 5B), but cold BeA did not compete with ^{3}H-NAA or ^{3}H-IAA transport (Fig. 5C). Cold oxIAA did not compete with ^{3}H-IAA in control or ABCB4-GFP cells (Fig 5E, F). 2-NOA decreased the net uptake of ^{3}H-2,4-D in control and PIN1-expressing cells, but had a lesser effect on the accumulation of 2,4-D in ABCB4-overexpressing cells (Fig. 7B). However, 2,4-D

uptake was enhanced in cells expressing ABCB4 after background auxin uptake was blocked with 2-NOA (Fig. 7C), suggesting a non-competitive interaction of 2,4-D with the ABCB4-based auxin efflux activity. This notion was further supported by the fact that pre-treatment of cells expressing ABCB4 with cold 2,4-D, but not cold IAA, resulted in an enhanced net uptake of ^{3}H-2,4-D (Fig. S5). An enhancement of auxin influx in BY-2 expressing ABCB4 was also observed following addition of NPA to 2-NOA-pretreated cells. This treatment resulted in increased 2,4-D accumulation, which is consistent with continued 2,4-D import by ABCB4 in combination with inhibition of the weak PIN and ABCB (including ABCB4) efflux activity (Fig. 7D). Hereafter, we observed gradual reversal activity of ABCB4 during ^{3}H-IAA accumulation (Fig. S6).

Our part of the work was supported by the Ministry of Education, Youth and Sports of the Czech Republic, project LC06034, and by the Grant Agency of the Czech Republic (project P305/11/0797).

6.6. Chapter 6 - New insights into auxin transport on cellular level by means of mathematical-modelling-motivated research

Petr Hošek*, **Martin Kubeš***, Martina Laňková, Petre I. Dobrev, Petr Klíma, Zdeněk Wimmer, Milada Kohoutová, Jan Petrášek, Eva Zažímalová, Klára Hoyerová, Marcel Jiřina

* These authors contributed equally to this work.

Manuscript in preparation

This manuscript originated from collaboration between Dr. Eva Zažímalová and Dr. Marcel Jiřina laboratories.

My contribution to this article was mainly in characterization of ^{3}H-BeA, ^{3}H-2,4-D and ^{3}H-NAA metabolism in tobacco BY-2 and Xanthi cell cultures together with identification of the main NAA metabolite in BY-2 cells as NAA glucosyl ester based on collaboration with Petre I. Dobrev (HPLC, LC-ESI-MS/MS analysis, confirmation of ester bond in NAA glucosyl ester and discussions), Zdeněk Wimmer (synthesis of NAA metabolite, discussion), Jiří Malbeck and Bedřich Pešek (MS analysis, discussions) and Helena Lipavská (HPLC analysis, discussion). I was partially involved in ^{3}H-2,4-D accumulation assays with auxin transport inhibitors NPA, CHPAA and their combination. I have been involved also in the manuscript preparation and processing of relevant results.

It was indicated that NAA is rapidly metabolized to the major metabolite (identified as NAA glucosyl ester) and after 10 min of incubation this metabolite was predominant in BY-2 cells. All NAA metabolites including predominant NAA glucosyl ester remained in cells and thus confused results of ^{3}H-NAA accumulation assays. Compared to NAA, 2,4-D was only partially metabolized to three unknown products and the analysis of incubation medium also confirmed retention of the 2,4-D metabolites in BY-2 cells. As a control BeA was used and surprisingly it was quickly metabolized to one predominant and one unknown minor products. In incubation medium both metabolites were detected insignificantly, which indicate that these products also remained in cells (Fig. 3A, B, C).
As mentioned above, the main NAA metabolite was identified by HPLC and MS techniques as NAA glucosyl ester (Fig. 4). Simultaneously the presence of ester bond in NAA glucosyl ester was confirmed by its incubation with NH_4OH (Fig. 5). Thanks to the weak 2,4-D metabolism, 2,4-D accumulation data measured under various conditions were used (in presence of auxin

transport inhibitors NPA or CHPAA, Fig. 6) and mathematical model of auxin transport to characterize and validate parameters of 2,4-D transport on cellular level was constructed.

The work was supported by the Ministry of Education, Youth and Sports of the Czech Republic project LC06034 and by the Grant Agency of the Czech Republic, project P305/11/0797.

7. Discussion

ABCB proteins as a plant orthologues of the mammalian multidrug-resistance proteins MDRs have been proved to participate in auxin transport (Martinoia et al., 2002; Verrier et al., 2008). Phenotypic defects caused by loss of ABCB function are most pronounced in vegetative organs and include dwarfism, curly wrinkled leaves, twisted stems and reduced apical dominance, supporting their role in auxin-based development (Bandyopadhyay et al., 2007). Auxin transport defects and dwarf phenotypes are more exaggerated in *Arabidopsis thaliana abcb1/abcb19* double mutants (Noh et al., 2001; Geisler et al., 2003). A direct role for ABCB1 and ABCB19 in cellular efflux was demonstrated when increased auxin retention was observed in mesophyll protoplasts from *Arabidopsis thaliana* and in heterologous systems of yeast, mammalian HeLa or tobacco BY-2 cells (Geisler et al., 2005; Petrášek et al., 2006). Similarly, as it was observed in case of PIN proteins, enhanced auxin efflux in HeLA or yeast cells with heterologously expressed *ABCB1* and *ABCB19* was inhibited by NPA (Geisler et al., 2005; Bouchard et al., 2006). However, contrary to PIN expression, the efflux mediated by the ABCBs in mammalian HeLa cells was insensitive to inhibitors of mammalian organic anion transporters (Geisler et al., 2005; Petrášek et al., 2006).

As shown in Chapter 1 of this thesis (Petrášek et al., 2006), we used inducible ABCB19-transformed BY-2 cell line to compare the roles of PINs and ABCBs in auxin efflux. The induction of ABCB19 led to a decrease in ^{3}H-NAA accumulation, similar to that observed in the PIN4, PIN6 and PIN7 lines. However, compared to PIN-mediated auxin efflux, the ABCB19-mediated NAA efflux was notably less sensitive to NPA. Whereas PIN-mediated transport was completely inhibited by NPA, about 20% of ABCB19-dependent transport was NPA insensitive. It is well known that NPA affinity chromatography was initially used to isolate the ABCB1, 4 and 19 proteins (Murphy et al., 2002; Geisler et al., 2003; Terasaka et al., 2005). Therefore, it is possible that NPA and other auxin efflux inhibitors are able to bind multiple binding and regulatory sites on various proteins with different affinities. Some evidence suggests that there are at least two distinct NPA-binding sites in *Arabidopsis thaliana* membranes. A high affinity site is associated with the inhibition of auxin transport at the PM and with an integral membrane protein. A low affinity site is thought to be a membrane-anchored or peripheral amidase (Murphy et al., 2002). Other non-specific sites of NPA action have been associated with membrane trafficking (Geldner et al., 2001). Moreover, auxin efflux inhibitors might have multiple effects, including modification of actin-based subcellular dynamics (Dhonukshe et al., 2008) or of the ABCB-PIN interaction (Blakeslee et al., 2007). Importantly, NPA has been shown to regulate ABCB-mediated auxin transport by influencing the interaction of ABCB with TWD1

immunophilin (Bailly et al., 2008; Wu et al., 2010) providing the mechanistic explanation for the various sensitivity of plant tissues to NPA.

To address whether PIN action *in planta* requires ABCB1 and ABCB19 proteins, the effect of *PIN1* overexpression on plant development was analyzed in *Arabidopsis abcb1/abcb19* double mutants (Petrášek et al., 2006). The *PIN1* overexpression led to pronounced defects in root gravitropism. Quantitative evaluation of reorientation of root growth revealed that *PIN1* overexpression in *abcb1/abcb19* had the same effects. These data showed that PIN1 action on plant development does not strictly require function of ABCB1 and ABCB19 proteins. This is also supported by evidence that PIN2 mediates auxin efflux in yeast, which is known to lack homologs to *Arabidopsis thaliana* ABCB proteins (Geisler and Murphy, 2006; Yang and Murphy, 2009). However, it was still unclear whether these two auxin transport machineries act *in planta* entirely independently or in a coordinated fashion.

This is partially addressed in the Chapter 2 of this thesis (Mravec et al., 2008), where the effect of increased auxin efflux in BY-2 cells was examined on the level of cell growth and morphology of non-induced and induced PIN7 and ABCB19 cell lines (already used in Petrášek et al., 2006). After induction of *PIN7* or *ABCB19* expression, analogous phenotypical changes occurred: cells ceased to divide, started to elongate, and formed and accumulated starch granules. Similar morphological changes were observed in induced PIN4 and PIN6 lines or constitutive PIN1 line which also showed increased auxin efflux. These results imply that the enhanced auxin efflux leads to depletion of auxin from cells, resulting in a change in the developmental program reflected by the switch from cell division to cell elongation. The auxin starvation phenotype in induced PIN7 cell line was rescued by the application of auxin efflux inhibitor NPA. However, similarly as shown in Petrášek et al. (2006) in less NPA-sensitive ABCB19 cell line, NPA was ineffective thus pointing to possible NPA-mediated regulation of ABCB19 with TWD1 (Bailly et al., 2008; Wu et al., 2010).

Further, results presented in Mravec et al. (2008) allowed propose the model for *Arabidopsis* roots, where ABCBs control auxin transport in auxin channel-forming cells and thus controlling the amount of auxin for directional PIN-dependent auxin transport. In the model, the proportions of ABCBs that do not colocalize with PINs act multilaterally in auxin efflux and thus regulate the effective cellular auxin concentration available for PIN-mediated transport. This combined action of PIN and ABCB determines how much auxin flows through auxin channels. Observation of a higher cellular auxin concentration in *abcb* mutants (Bouchard et al., 2006) that might enhance PIN-mediated transport directly supports this scenario. It is likely that for long-distance transport, e.g. in stems, another mode of ABCB and PIN interaction functions as suggested by strong auxin transport defects in *abcb* mutant stems (Noh et al., 2001; Geisler et al.,

2005). It is important to note that different internal or external cues, such as light, can influence the extent and mode of PIN-ABCB interactions, for instance at the level of functional pairing of PINs and ABCBs (Blakeslee et al., 2007; Titapiwatanakun et al., 2009). Also, the activity of previously uncharacterized ABCBs may significantly contribute to auxin transport. In summary, our model could provide an explanation of the existence of two auxin transport mechanisms (PIN-based and ABCB-based) that ensure precise and proper formation of spatial and temporal auxin distribution in plants.

Like the PIN proteins, ABCBs exhibit both the polar and apolar distribution on the PM. Both ABCB1 and ABCB4 exhibit polar localisations in elongating and mature epidermal and/or cortical root cells and apolar localisations in the lateral root cap (Geisler et al., 2005; Terasaka et al., 2005; Cho et al., 2007). ABCB19 exhibits a predominantly polar localisation on the plasma membrane in immature vascular cells and apolar distribution in mature vascular, cortical and epidermal cells at the root apex (Blakeslee et al., 2007; Wu et al., 2007). Blakeslee et al. (2007) showed by comparisons with the cytokinesis marker KNOLLE that the polar localisation of ABCB19 seen in immature vascular tissues coincides with cell wall maturation, but not with early cell plate formation. In Chapters 1, 3, 4 and 5 (Petrášek et al., 2006; Jelínková and Malínská et al., 2010; Laňková et al., 2010) of this thesis the localization of ABCB19 and ABCB4 is characterized in BY-2 cells. For ABCB19 (Petrášek et al., 2006) the localization was shown to be not predominantly at transversal plasma membranes within the cell chain as it was shown for PIN proteins. This is in agreement with mostly non-polarly localized ABCB19 in various tissues of *Arabidopsis thaliana* (Petrášek and Friml, 2009). Moreover, in Chapter 3 (Jelínková and Malínská et al., 2010), ABCB4-transformed cell line was shown to be transiently re-localized from the plasma membrane with routinely used concentrations of FM 4-64 and FM 5-95. The active process of re-localization is blocked neither by inhibitors of endocytosis (BFA - which in plants act as an inhibitor of anterograde vesicle trafficking) nor by cytoskeletal drugs (wortmannin - which have all been successfully shown to block endocytosis in plants (Emans et al., 2002; Dettmer et al., 2006)). The formation of FM-induced ABCB4 patches was blocked only after sodium azide treatment, which suggests that the process of FM-induced PM protein re-localization is energy-dependent, possibly via an ATP-driven step.

Interestingly, in Laňková et al. (2010) we showed that after the application of putative auxin transport inhibitors 1-NOA, 2-NOA and CHPAA, ABCB4 is more stable on PM than AUX1 or PIN1 proteins. While in AUX1- and PIN1-transformed BY-2 cell lines fast redistribution of proteins from PM inside the cells was induced, ABCB4 in form of a few membrane aggregates appeared only after long inhibitors treatment. Both NOAs (1-NOA being more effective) can induce the formation of endosomes containing auxin carriers. At least partly

this may be the reason for the decrease in auxin influx or efflux after the 1-NOA or 2-NOA treatments. The fact that NOA was not able to induce fast redistribution of ABCB4 (as observed for AUX1 and PIN1) and that this ABCB4 appeared in membrane aggregates after longer treatment suggests a membrane composition-dependent NOA effect. The most important in this respect seems to be sterol composition, namely the presence of sterols and sterol-associated proteins (Borner et al., 2005); since ABCB4 was reported to be present in these compartments (Titapiwatanakun and Murphy, 2009), thus the ABCB4 could be more resistant to 1-NOA treatment.

Further in Chapter 5 (Kubeš and Yang et al., submitted), we provided evidence that ABCB4 functions as a homeostatic switch on the PM for regulation of cellular auxin levels. We confirmed that ABCB4 function in shootward auxin movement from the root apex increases with auxin concentration, but it is evident in regulating root hair elongation at lower concentrations. It seems that ABCB4 plays a more important role in controlling auxin movement out of the elongation zone as previously proposed (Peer and Murphy, 2007). Expression of *Arabidopsis thaliana* ABCB4::ABCB4-GFP in BY-2 resulted in abundance and localisation similar to that reported for *35S*-driven expression of ABCB4 in BY-2 (Cho et al., 2007) as well as a partial inhibition of cell division compared to untransformed cells. This inhibition was similar to *PIN1pro*:PIN1-GFP expression, but it was less severe than in auxin-starved BY-2 cells or in cells expressing *Arabidopsis thaliana PIN4*, *PIN6*, *PIN7* and *ABCB19* (Petrášek et al., 2006; Mravec et al., 2008). Further, cell elongation observed with expression of *Arabidopsis thaliana ABCB19* or *PIN1* (Mravec et al., 2008) was not observed in 3-day ABCB4-GFP cells, perhaps due to decreased transversal PM localisation of ABCB4-GFP (Cho et al., 2007) compared to PIN1-GFP. This decrease in transversal localisation may be a consequence of faster lateral diffusion or trafficking of ABCB4 compared to PIN1-GFP as indicated by FRAP analysis. As reported previously (Petrášek et al., 2002, 2006), PIN1 expression in BY-2 resulted in increased ^{3}H-NAA efflux, and NPA treatment increased NAA retention. Similarly, BY-2 cells expressing *ABCB4pro*:ABCB4-GFP exhibited increased retention of NAA after NPA treatment, which is in agreement with results in BY-2 cells overexpressing *ABCB4* under the control of a *35S* promoter (Cho et al., 2007). 2,4-D is often used as a substitute for IAA in growth assays, as it is highly stable (Delbarre et al., 1996) and is poorly distributed through the plant by auxin polar transport mechanisms (Pitts et al., 1998). This synthetic auxin taken up via AUX1/LAX permeases (Bennett et al., 1996; Yang et al., 2006; Yang and Murphy, 2009) was shown to be an uptake substrate for ABCB4 expressed in *Schizosaccharomyces pombe,* and to non-competitively inhibit ABCB4 ^{3}H-IAA efflux activity (Yang and Murphy, 2009). 2,4-D is a weak efflux substrate for all ABCB and PIN proteins expressed in non-plant heterologous systems, but it is a preferred

efflux substrate for the ABCG37/PDR9 pleiotropic substrate transporter (Titapiwatanakun et al., 2009; Růžička et al., 2010). Our analysis of 2,4-D effects on root hair elongation confirmed that this compound is an ABCB4 uptake substrate *in planta*. Low concentrations of 2,4-D compensated for differences in *abcb4* root hair elongation in a manner similar to what is seen with IAA, and higher 2,4-D concentrations inhibited root hair elongation equally in both *abcb4* and wild-type. However, unlike IAA, an intermediate concentration of 2,4-D (50 nM) inhibited wild-type root hair elongation to a greater extent than in *abcb4*, suggesting a direct role of ABCB4 in 2,4-D uptake. This result cannot be attributed to enhanced AUX1-driven uptake in *abcb4* root hairs, as *AUX1* is not expressed in trichoblasts (Jones et al., 2009). 2,4-D is also the preferred substrate for native 2-NOA-sensitive auxin influx assays in BY-2, and ABCB4 is resistant to 2-NOA inhibition (Laňková et al., 2010). As expected, 2-NOA decreased the net uptake of ^{3}H-2,4-D in control and PIN1-expressing cells, but had a lesser effect on the accumulation of 2,4-D in ABCB4-overexpressing cells. However, 2,4-D uptake was enhanced in cells expressing *ABCB4* after background auxin uptake was blocked with 2-NOA, suggesting a non-competitive interaction of 2,4-D with the ABCB4-based auxin efflux activity. This notion was further supported by the fact that pre-treatment of cells expressing *ABCB4* with cold 2,4-D, but not cold IAA, resulted in an enhanced net uptake of ^{3}H-2,4-D. An enhancement of auxin influx in BY-2 expressing ABCB4 was also observed following addition of NPA to 2-NOA-pretreated cells. This treatment resulted in an increase in 2,4-D accumulation, consistent with continued 2,4-D import by ABCB4 in combination with inhibition of the weak PIN and ABCB (including ABCB4) efflux activity. One explanation for the lack of ^{3}H-NAA efflux seen in BY-2 cells expressing ABCB4 might be that the cells are routinely maintained on medium containing the synthetic auxin 2,4-D (Nagata et al., 1992). Although routinely washed out with uptake buffer before auxin transport assays are conducted (Cho et al., 2007), 2,4-D from the BY-2 cell culturing medium bound to ABCB4 may be difficult to completely eliminate, especially as 2,4-D is poorly metabolized in tobacco cells compared to IAA and NAA (Delbarre et al. 1994, 1996). In short term yeast assays, ABCB4 showed influx activity at lower IAA concentrations and ABCB4 transport activity switched to efflux at concentrations >250 nM or after longer exposure to IAA. Conversion to efflux rather than conventional saturation suggests that IAA uptake mediated by ABCB4 is an indirect effect of transporter activity and suggests that ABCB4 could mobilise other substrates. Our results presented here indicate that ABCB4 regulates cellular auxin levels in the *Arabidopsis thaliana* root epidermis by enhancing auxin uptake when cellular levels are low and catalyzing auxin efflux when internal levels rise. Alternatively, the combination of more hydrophobic anchoring and additional IAA binding sites in ABCB4 may provide a mechanism for substrate uptake that would not occur in ABCB1 or 19 during the

conformational change associated with the first of two ATP hydrolysis steps required for ABC transport function (Knöller and Murphy, 2010). Substrate activation and conformational change has been shown for other ABC transporters (Loo et al., 2003; Terasaka et al., 2005; Sauna et al., 2008), and apparent drug-induced reversal of mammalian ABCB1 and ABCG22 activity has recently been reported (Shi et al., 2011). Finally, we confirmed that ABCB4 enhances 2,4-D uptake, but not efflux, and thus ABCB4 appears to be a direct herbicidal target of 2,4-D. Binding of 2,4-D to ABCB4 results in increased accumulation of both 2,4-D and other auxins in root epidermal cells and so it is likely to amplify the herbicidal effects of the compound. As such, tissue-specific manipulation of *ABCB4* expression may be a useful tool for decreasing damage to crop plants caused by 2,4-D drift.

The mathematical modelling of auxin transport at the cellular level revealed and confirmed the parameters for better and more precise characterization of auxin transport mechanisms in tobacco BY-2 cells. In Chapter 6 (Hošek and Kubeš et al., in preparation), we indicated that NAA is strongly and rapidly metabolized during accumulation experiments in BY-2 cells. We identified the predominant metabolite as NAA glucosyl ester (NAA-Glc). Further, we showed that NAA-Glc is retained in the cells, thus raising apparent intracellular concentration of NAA previously measured during accumulations of radiolabeled auxins. This might have led to serious underestimation of auxin efflux carriers transport capacity for NAA as well as IAA in the past. In contrast, metabolism of 2,4-D remains fairly weak in the time span of accumulation assays, making 2,4-D an ideal tool for probing auxin transport on cellular level, at least on the level of auxin uptake. Hence, using 2,4-D accumulation data measured under various conditions (in presence of auxin transport inhibitors NPA or CHPAA) and employing mathematical model of auxin transport we characterize and validate parameters of 2,4-D transport on cellular level.

Obtained experimental data provides a basis for mathematical description of characteristics of transport of particular auxins, including their metabolism. Making use of relevant mutants and overexpressors, this data can also serve as a basis for further modelling related to contributions of particular auxin transport proteins and for construction of detailed mathematical model of auxin transport on the cellular level.

8. Conclusion

In the papers and manuscripts forming this thesis it was shown that:

- The overexpression of ABCB19 results in a decrease of ^{3}H-NAA accumulation in BY-2 cells, similar to that observed in the PIN7-overexpressing line, and produces the auxin starvation phenotype.
- Compared to PIN7-mediated auxin efflux, the ABCB19-mediated NAA efflux is less sensitive to auxin efflux inhibitor NPA, and the ABCB19-dependent auxin starvation phenotype cannot be rescued by NPA.
- Auxin transporters ABCB1, ABCB4 and ABCB19 display non-polar localization on PM in BY-2 cells.
- ABCB4 protein is more stable and resistant to auxin transport inhibitors 1-NOA, 2-NOA and CHPAA in the plasma membrane of BY-2 cells.
- Routinely used concentrations of FM styryl dyes FM 4-64 trigger transient re-localization of ABCB4.
- ABCB4 functions as a homeostatic switch on the PM for regulation of cellular auxin levels. There is a non-competitive interaction of 2,4-D with the ABCB4-based auxin efflux activity. ABCB4 enhances 2,4-D uptake, but not efflux, and ABCB4 appears to be a direct herbicidal target of 2,4-D.
- NAA is strongly and rapidly metabolized during accumulation assays in BY-2 cells and the predominant metabolite is NAA glucosyl ester, which is retained in the cells. In contrast, metabolism of 2,4-D remains fairly weak in the time span of accumulation assays, making 2,4-D good tool for probing auxin transport on cellular level. Using 2,4-D accumulation data measured under various conditions and exploring the modified mathematical model of auxin transport, the parameters of 2,4-D transport on cellular level were characterized and validated.

Altogether, results presented in this thesis further clarified the role of ABC-transporters in translocation of auxin across plasma membrane and pointed at their involvement in establishment and maintenance of cellular auxin homeostasis in a way different from that of PIN5.

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