AUXIN BINDING PROTEIN 1 (ABP1)
and its role in the auxin management in plant cells

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This is to certify that this thesis is not a subject of any other defending procedure. It contains set of original results that have been published or are going to be published in the international peer-reviewed scientific journals.

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Summary

Auxin, represented by its predominant form indole-3-acetic acid (IAA), is the major plant hormone that co-ordinates the development of plant body as well as the reaction to various developmental and environmental stimuli. The broad range of auxin-triggered responses is achieved by auxin concentration gradients that are formed by processes of auxin biosynthesis, metabolism and transport. In principle, there exist two levels of responses: fast mostly non-genomic one and the slower one that include modification(s) of gene expression. The molecule of auxin entering the cell is supposed to bind to its receptor, and the process further continues by cascade of reactions inside the cell and consequently in the tissue and the plant organ. Besides well described function of auxin receptors from TRANSPORT INHIBITOR RESPONSE1/AUXIN-BINDING F-BOX PROTEIN (TIR1/AFB) family triggering specifically auxin-driven, proteasome-mediated gene expression there is another known putative auxin receptor AUXIN BINDING PROTEIN 1 (ABP1) considered to mediate rather non-genomic reactions, which can precede the change in gene transcription leading to the reprogramming of cell fate. ABP1 as an auxin receptor can be involved in every aspect of the regulation of auxin responses, metabolism and transport.

In this thesis, the role of ABP1 in the auxin management in plant cells was followed using simplified model material of suspension-cultured cells of tobacco BY-2 cell line. BY-2 is commonly cultured with high multiplication rate in the cultivation medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin that is not a good ligand for ABP1. The silencing of *ABP1* expression using *ABP1* gene in the antisense orientation did not totally prevent cell division activity of BY-2 cells, but the cells were not able to expand suggesting the role of ABP1 in cell elongation. The immunolocalization of ABP1 further showed that it is preferentially localized at the plasma membrane in elongating cells, while in dividing cells it is localized at the endoplasmic reticulum. Moreover, cells overexpressing *ABP1* were less sensitive to the inhibitor of cellular auxin efflux 1-naphthylphthalamic acid (NPA) suggesting the involvement of ABP1 in the active transport of auxin from cells. This finding was tested further by auxin transport assays in double transformants carrying both ABP1 and the auxin efflux carriers from PINFORMED (PIN) family that clearly documented the ABP1-mediated PIN-dependent auxin transport. To study the mechanism of ABP1-triggered changes in PIN-dependent auxin transport, *in vivo* confocal microscopy observation of PIN1-GFP dynamics after *ABP1* over-expression was performed. The fluorescence recovery after photobleaching (FRAP) revealed that conditional *ABP1* overexpression

stimulated the endocytosis of PIN1 suggesting negative regulation by ABP1 of the plasma membrane pool of active auxin efflux carriers. Opposite effect was observed in cells treated with high auxin concentration. High auxin levels were earlier reported to block PIN endocytosis thus promoting auxin transport from cells. These observations together with the FRAP experiments suggest that ABP1 plays an important role in regulation of endocytosis and helps to retain PINs on plasma membrane. The analysis of products of plasma membrane-associated phospholipases suggested that their activity, previously reported to be important for the trafficking of PINs, is modified after *ABP1* over-expression. Besides the role of plasma-membrane-localized ABP1 in the intercellular auxin transport, this thesis brings also the data on the role of ABP1 in the intracellular auxin management. By the analysis of cell growth parameters and intracellular IAA metabolic profiling, ABP1 is shown to rescue the effect of over-expression of the endoplasmic-reticulum-localized PIN5, namely induced cell death and promoted IAA conjugation. Thus, ABP1 is likely to contribute to maintenance of free IAA pool by preventing its conjugation.

Based on the results presented here, it is concluded that ABP1 mediates cell-to-cell transport of auxin, dependent on the plasma-membrane-localized PIN proteins. Moreover, ABP1 is also an important player in the maintenance of intracellular IAA homeostasis.

Souhrn

Auxin, reprezentovaný především kyselinou indol-3-octovou (IAA), je hlavním hormonem, který koordinuje vývoj rostliny a zprostředkuje odpovědi jak na různé vývojové podněty tak na vlivy prostředí. Široká škála auxinem řízených odpovědí je dosahována pomocí auxinových koncentračních gradientů, k jejichž vytvoření přispívá biosyntéza auxinu, jeho metabolismus a transport.

Odpovědi na auxinem nesený signál probíhají na dvou hlavních úrovních: rychlé, většinou negenomické odpovědi a ty, které změnu genové exprese zahrnují. Předpokládá se, že molekula auxinu se při vstupu do buňky váže na receptor a spouští tak řetězec reakcí odehrávajících se uvnitř buňky, které jsou pak následovány změnami na úrovni pletiv a orgánů.

Kromě poměrně dobře popsané funkce auxinových receptorů z rodiny TRANSPORT INHIBITOR RESPONSE1/AUXIN-BINDING F-BOX PROTEIN (TIR1/AFB), jejíž členové zprostředkují auxinem řízenou genovou expresi, je dlouho znám ještě jeden receptor pro auxin - AUXIN BINDING PROTEIN 1 (ABP1), jenž zřejmě zprostředkuje spíše negenomické reakce, které ale také mohou předcházet změně transkripce genů a vést až ke změně osudu buňky. Jakožto receptor pro auxin, ABP1 může zasahovat do všech procesů regulace auxinových odpovědí, včetně metabolismu a transportu.

Předmětem této práce je poznání způsobu, jakým ABP1 ovlivňuje hospodaření s auxinem v buňce. Jako zjednodušený modelový materiál byla využita tabáková suspenzní kultura BY-2. Buňky linie BY-2 jsou běžně pěstovány v médiu se syntetickým auxinem kyselinou 2,4-dichlorfenoxyoctovou (2,4-D), která není příliš dobrým ligandem pro ABP1. Buňky s utišenou expresí genu *ABP1* neztrácejí schopnost se dělit, ale neprodlužují se, což naznačuje, že ABP1 je důležitý pro buněčnou expanzi. Lokalizace ABP1 pomocí protilátky ukázala, že v dělících se buňkách se ABP1 nachází především v endoplazmatickém retikulu, zatímco v prodloužených buňkách se vyskytuje na plazmatické membráně. Navíc buňky se zesílenou expresí *ABP1* jsou méně citlivé ke kyselině 1-naftylftalamové (NPA), inhibitoru exportu auxinu z buňky, což naznačuje úlohu ABP1 také v regulaci mezibuněčného toku auxinu. Toto zjištění bylo ověřeno pomocí měření transportu auxinu v buněčné linii exprimující ve zvýšené míře ABP1 společně s přenašečem auxinu ven z buňky z rodiny proteinů PIN-FORMED (PIN) a bylo potvrzeno, že ABP1 reguluje export auxinu z buněk zprostředkovaný PINy. Způsob, jakým ABP1 ovlivňuje aktivitu PINů, byl zjišťován s využitím konfokálního mikroskopu sledováním dynamiky proteinů PIN1-GFP po indukci

overexprese *ABP1*. Metodou „fluorescence recovery after photobleaching“ (FRAP) bylo zjištěno, že ABP1 podporuje endocytózu PINů, a snižuje tak počet aktivních přenašečů PIN na plazmatické membráně. Opačný projev byl pozorován u buněk v médiu se zvýšenou koncentrací auxinu. Již dříve bylo popsáno, že vyšší množství auxinu vede ke zpomalení endocytózy a ke zvýšenému transportu auxinu ven z buňky. Toto pozorování spolu s experimenty FRAP naznačují, že ABP1 hraje důležitou roli v regulaci procesu endocytózy a pomáhá zadržení PINů na plazmatické membráně. Bylo prokázáno, že některé fosfolipázy asociované s plasmatickou membránou se účastní regulace transportu váčků nesoucích PIN-proteiny. Pomocí analýzy produktů enzymového štěpení těmito fosfolipázami bylo ukázáno, že ABP1 ovlivňuje také aktivitu těchto enzymů.

Kromě toho, že bylo ukázáno, že ABP1 umístěný na plazmatické membráně má významnou úlohu v mezibuněčném transportu auxinu, jsou v této práci uvedena také data, naznačující na roli ABP1 ve vnitrobuněčném hospodaření s auxinem. S využitím analýzy růstových parametrů buněk BY-2 a intracelulárních metabolických profilů nativního auxinu IAA bylo prokázáno, že ABP1 zabraňuje projevům zvýšené exprese proteinu PIN5, která vede ke zvýšené úmrtnosti buněk. Transportér PIN5 je umístěn na membráně endoplazmatického retikula a podporuje převedení volné IAA do formy konjugátů. ABP1 tomuto předchází a přispívá tak k udržení hladiny volné IAA.

Na základě výsledků zde uvedených lze usuzovat, že ABP1 moduluje tok auxinu mezi buňkami zajišťovaný transportéry typu PIN. Současně je ABP1 důležitý i pro udržení intracelulární homeostáze auxinu.

1 Outlines

ABP1 is a putative auxin receptor, whose importance for the plant development has been documented during embryogenesis (Chen et al., 2001b) as well as during postembryonic development of shoot (Braun et al., 2008) and root (Tromas et al., 2009). Although there exists detailed information about its structure, expression patterns along plant body and localization within the cell, we have still only fragmented understanding of its physiological function. Despite the predominant localization of ABP1 in the lumen of ER (Henderson et al., 1997; Feckler et al., 2001), the function of this receptor seems to be held on plasma membrane. The plasma membrane electric potential measurements (Leblanc et al., 1999b) and protoplast swelling assays (Steffens et al., 2001) confirmed the plasma membrane ABP1 role during cell expansion. Leaf cells of tobacco plants over-expressing ABP1 were reported to expand upon exogenous auxin treatment as well (Jones et al., 1998). Although protoplasts are suitable for single cell level experiments focused on the plasma membrane characteristics, they are rather artificial system. This thesis uses as the experimental material suspension cultures of tobacco that provide some important characteristics to study cellular aspects of ABP1 role in the auxin management. Using synchronized tobacco suspension culture BY-2 was already demonstrated that ABP1 is crucial for auxin regulation of the cell cycle (David et al., 2007). Cell line of *Nicotiana tabacum* L. cv. Bright Yellow 2 (Nagata et al., 1992) is highly homogenous and shows exceptionally high growth rate. One subcultivation interval is typically 7 days long. At the beginning of the subcultivation period the population consists mainly from elongated single cells or cell chains with 2-3 cells. Shortly after the inoculation (6-12 h) cells start to divide and this exponential phase lasts 5-6 days. During this phase, several waves of cell division produce cell chains, where individual cells are not elongating. The patterning of cell division is similar as described for another tobacco cell line cv. Virginia Bright Italia, VBI-0 (Campanoni et al., 2003) with always higher number of chains with even number of cells (2, 4, 6, 8) than with odd number of cells (1, 3, 5, 7). Multicellular cell files are exceptional and their higher occurrence might indicate impaired auxin control of the cell cycle. The end of the subculture interval is characterized by the decreasing intensity of cell division and accelerated cell elongation with disintegration of cell files.

The role of ABP1 in the processes of cell division and cell expansion is addressed in the first part of this thesis in BY-2 cells transformed constitutively with cDNA of tobacco *ABP1* gene in both sense and antisense orientation to obtain lines with over-expressed or down-regulated ABP1. Moreover, inducible heterologous inducible expression of Arabidopsis *ABP1*

has been used as well. These lines were used to study the role of ABP1 in cell division and expansion. Growth characteristics of suspension cells (cell length categories, number of cells in cell chains) as well as cell phenotype and division rate were compared with non-transformed control cells. The effect of ABP1 over-expression or down-regulation was further studied after the manipulation with auxin levels in the cultivation medium. BY-2 cells are routinely propagated in MS medium supplemented with 1μM 2,4-D, which is needed for cell division, but it is not good substrate for ABP1-mediated physiological effects such as cell expansion that is more likely stimulated by IAA. Therefore, localization of tobacco ABP1 in both exponential and elongating cells was studied with indirect immunofluorescence method. cDNA of tobacco ABP1 was expressed in *Escherichia coli*, protein purified and polyclonal serum raised in rabbit. This antibody was used to visualize ABP1 in 2,4-D and IAA-treated cells.

Besides studying the ABP1 role in cells with various supply of exogenous auxin, further analysis presented in this thesis is focused on the testing of the role of ABP1 in the cell-to-cell auxin transport. To address this question the inhibitors with similar inhibitory impact on auxin transport, particularly the 2-(1-pyrenoyl) benzoic acid (PBA) affecting actin dynamics (Dhonukshe et al., 2008) and phytotropin NPA (Katekar and Geissler, 1980) have been used in transformed cell lines. Moreover, ABP1 has been transformed into cells carrying inducible PIN7, a plasma membrane protein that has been shown to play a rate-limiting function in cellular auxin efflux (Petrášek et al., 2006). In these cells, the role of ABP1 in PIN-dependent auxin transport using auxin accumulation assays and phenotypic studies has been studied. For all auxin accumulation assays BY-2 cells were equilibrated before experiment and radioactively labeled auxin was added to the cells at time zero. During the uptake period the cells were harvested, extracted in ethanol and radioactivity determined by liquid scintillation counting. For all auxin transport experiments 2nM [^{3}H]NAA was used being a good substrate both for the ABP1 (Löbler and Klämbt, 1985a) and for the auxin efflux machinery in tobacco cells (Delbarre et al., 1996).

To uncover, at least partially, the mechanisms by which ABP1 affects the auxin transport across membrane, *in vivo* confocal microscopy of PIN1-GFP was performed. BY-2 cells line over-expressing constitutively plasma membrane auxin carrier from Arabidopsis PIN family in GFP fusion (PIN1-GFP) and inducible Arabidopsis ABP1 was used to monitor the effect of ABP1 on PIN protein intracellular dynamics. PINs undergo dynamic recycling between plasma membrane and endosomal compartments (Kleine-Vehn et al., 2008). This recycling is crucial in the redirection of auxin flow during plant development (for review see Kleine-Vehn

and Friml, 2008). The retrograde trafficking of PINs is clathrin-dependent (Dhonukshe et al., 2007) and depends on sterol composition of plasma membrane (Willemsen et al., 2003; Men et al., 2008). The polar delivery of PINs to plasma membrane is regulated by guanine nucleotide exchange factor for ADP-ribosylation factors (ARF-GEF) (Kleine-Vehn et al., 2008) and their localization to apical or basal end also depends on their phosphorylation status (Michniewicz et al., 2007). Auxin is known to inhibit PIN endocytosis thus promoting its own transport (Paciorek et al., 2005). Although the mechanism of this rather non-genomic auxin effect is not fully understood, it seems that ABP1 might be involved in this process (Robert et al., 2010). Therefore, the regulation of PIN dynamics and vesicle trafficking might belong among these non-genomic rapid auxin responses that are assisted by ABP1. By this mechanism ABP1 might affect the abundance of PIN proteins at the plasma membrane and thus to control auxin efflux from the cell. In this thesis, a technique of measuring a fluorescence recovery after photobleaching (FRAP) in PIN1-GFP cells after inducible overexpression of ABP1 was used. The value of GFP signal intensity reflects the number of GFP-tagged proteins present at the plasma membrane at certain time as they are dynamically releasing and fusing. The application of the inhibitor of anterograde vesicle trafficking brefeldin A (BFA) or the inhibitor of endocytosis tyrphostin A23 (Tyr A23) allowed us to discriminate whether ABP1 affects retro- or anterograde vesicle trafficking. Tyr A23 impairs the clathrin-dependent endocytosis as it prevents cargo recruitment into the endocytic vesicle; therefore PINs are retained at the plasma membrane (Dhonukshe et al., 2007). BFA inhibits vesicle trafficking from endosomes to the plasma membrane by the inhibition of activity of GNOM, an ARF-GEF (Geldner et al., 2003).

Since the physiological role of ABP1 during cell expansion was shown to occur on plasma membrane (Rück et al., 1993; Leblanc et al., 1999a) it is expected that upon binding of auxin, ABP1-triggered signal for PIN recruitment to the endocytotic vesicles is likely to take place at the plasma membrane as well. Therefore, the involvement of activity of phospholipases could be expected. Three types of phospholipases were shown to be related to auxin signaling. The phospholipase A2 (PLA2), which regulates auxin driven gene expression (Scherer et al., 2007) and has been recently shown to be important for PIN protein trafficking to the plasma membrane (Lee et al., 2010), phospholipase Dζ2 (PLDζ2), which expression is enhanced by auxin and it is required for PIN trafficking (Li and Xue, 2007) and phosphatidyl-hydrolysing phospholipase C (PC-PLC) that was also shown to interact with PIN-dependent auxin transport in oat shoots (Yun et al., 2006). Generally, phospholipases are effector enzymes catalyzing hydrolysis of various compounds, among other things

phosphatidylcholine, to specific products, which then may serve as second messengers for various signaling cascades. Products of the three above mentioned types of phospholipases have been studied using high performance thin layer chromatography (HP-TLC). Fluorescently labeled phosphatidylcholine as a substrate was added to BY-2 cells over-expressing tobacco ABP1 alone or together with PIN7, both in the presence or absence of auxin. This approach allowed answering the question whether the auxin signal may be passed from ABP1 *via* the phospholipases to control vesicle trafficking of PIN proteins.

Besides well-characterized plasma membrane PINs mediating cell-to-cell auxin transport there are three other PINs in Arabidopsis genome that have much shorter cytosolic loop. One of them, PIN5, residing at the membrane of endoplasmic reticulum (ER) has been shown to facilitate auxin uptake into the lumen of ER. Since the ER contains enzymic apparatus for the auxin conjugation it has been suggested that PIN5 contributes to the regulation of auxin homeostasis (Mravec et al., 2009). Interestingly, Chen et al. (2006) observed higher auxin metabolism and the conjugation in particular, in ABP1 antisense BY-2 cells. This indicates that ABP1 is also essential for the maintenance of auxin homeostasis by preventing the auxin conjugation and keeping certain pool of free IAA for immediate usage. Therefore, cell growth parameters and IAA metabolic profiling have been determined in BY-2 double transformants carrying inducible *PIN5* gene and constitutive tobacco *ABP1* gene. *PIN5* overproduction was reported to induce cell death, probably by enhanced auxin transport into the ER (Skůpa et al., 2008) and therefore the ABP1 is expected to act against this phenomenon. The viability of tobacco cells was assessed in the medium supplied with two different auxins with different specificity to ABP1 to address more precisely its function in cell mortality protection. In parallel, IAA metabolic profiles in these cell lines helped to reveal whether ABP1 contributes to or protects the IAA conjugation. Radioactively labeled IAA was added to the cells and after certain time the extract containing original IAA and its products was isolated and separated with high performance liquid chromatography (HPLC).

2 Objectives

Based on the outlines described above, there are four major objectives of this thesis:

1) To characterize the role of ABP1 in cell division and cell expansion
2) To prove the involvement of ABP1 in the intercellular auxin transport
3) To understand the mechanism of ABP1-triggered changes in PIN-dependent auxin transport
4) To examine the role of ABP1 in the intracellular auxin management

These objectives has been addressed using tobacco BY-2 cells by the inducible or constitutive expression of *ABP1* gene in sense and antisense orientation with subsequent observation of cell growth parameters, by ABP1 immunolocalization, by the co-expression of *ABP1* and plasma membrane and endoplasmic reticulum-localized PIN auxin carriers and subsequent analysis of auxin transport and metabolism, by *in vivo* confocal microscopy observation of PIN proteins dynamics and determination of products of plasma membrane-localized phospholipases.

3 Introduction

3.1. Auxin, plant hormone with many physiological and developmental effects

Auxin, typically indole-3-acetic acid (IAA), is involved in almost every developmental process of plants. As early as in 19th century Charles Darwin and his son (1880) revealed that the grass coleoptiles are able to grow towards directed light source. Since that time auxin was revealed to act in embryo and fruit development, organogenesis, apical dominance, vascular tissue differentiation, root patterning, root hair formation, elongation and tropic growth including shade avoidance response, as well as defense response to the pathogens (reviewed in Alabadi et al., 2009; Vanneste and Friml, 2009).

To evoke specific physiological and developmental changes auxin interacts with individual cells within plant tissue where it induces certain responses. Nonetheless, not just a simple auxin occurrence plays an important role in plant development processes. Actually, auxin functions via formation of auxin gradients established by co-action of auxin biosynthesis, metabolic changes and intercellular auxin transport. These in turn are controlled by the feed-back auxin-driven gene expression and other endogenous and environmental signals (Figure 3.1).

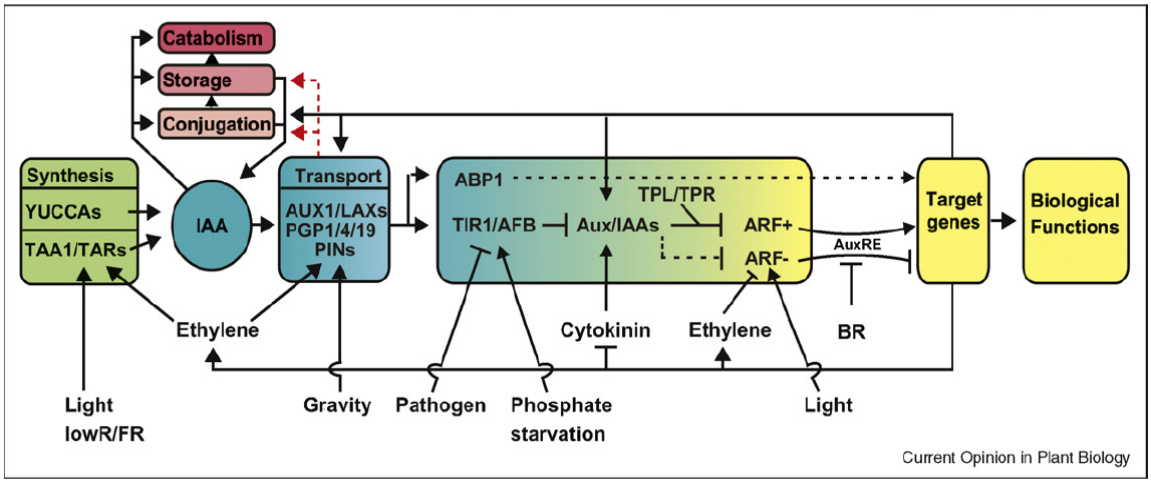

Figure 3.1: The modulation of auxin metabolism, transport and response by endogenous and environmental signals, from Kieffer et al. (2010).

3.2. The mechanism of auxin action

All auxin signaling pathways necessarily begin from the perception of auxin molecule by a receptor followed by cascade of chemical-physical reactions resulting in a spectrum of physiological outcomes. Generally, auxin triggers signaling pathways of genomic or non-

genomic nature. The non-genomic reactions, that can be very fast, occur predominantly on the level of plasma membrane. The auxin-triggered gene expression occurs in the nucleus and takes from few minutes to many hours. Despite the secondary auxin-induced genes, whose expression starts after tens of minutes and persists for many hours, the early auxin-induced response proteins are detectable after around 10-15 minutes of auxin application (reviewed in Napier and Venis, 1995; Guilfoyle and Hagen, 2001). Among non-transcriptional reactions to auxin belongs the activation of H^+ATPase extruding protons into cell wall thus causing cell wall loosening, plasma membrane electric potential changes, decrease in plasma membrane microviscosity and Ca^{2+} release from vacuole and ER to the cytosol (reviewed in Brummell and Hall, 1987; Badescu and Napier, 2006). There exist also other processes which are faster than can be explained by changes in the corresponding gene transcription. Auxin promotes phospholipase A (PLA) activity within 2 min(Scherer and Andre, 1989), activates MAP kinase within 5 min (Mockaitis and Howell, 2000) and the activation of rac-like GTPases occurs within 5 min (Tao et al., 2002). Also the auxin controlled inhibition of endocytosis is achieved during less then 10 minutes (Paciorek et al., 2005). All protein components of these pathways pre-exist in target cells prior to the stimulus hence these 'fast' pathways are unlikely dependent solely on auxin receptors such as *transport inhibitor response 1* (TIR1) and *auxin signaling F-box* (AFB) protein family, triggering mostly rapid gene expression (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

Nevertheless, transcription of auxin early responsive genes can be detected within 5 minutes, including genes related to soybean (*Glycine max*) *GH3* first isolated from excised hypocotyls, the *SAUR* (Small Auxin Upregulated RNAs) and the *auxin/indole-3-acetic acid* (*Aux/IAA*) genes. *Aux/IAAs* encode mostly rapidly turned-over repressors of auxin response factors (ARF), the transcription factors binding to the auxin-responsive elements (AREs) in promoters of auxin-driven genes. ARFs can either activate or repress transcription of the target gene (reviewed in Hagen and Guilfoyle, 2002). There are 23 members of ARF family and 29 Aux/IAA transcription factors in *Arabidosis thaliana* (Liscum and Reed, 2002). Through the Aux/IAA-ARF system plant can achieve many combinations leading to a broad range of developmental outcomes. Auxin controls the abundance of Aux/IAA proteins by promoting their rapid ubiquitin-mediated proteolysis thereby relieving repression of ARF activity. Auxin enhances the interaction of Aux/IAAs with members of the TIR1/AFB family of auxin receptor F-box proteins (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). These F-box proteins are part of the ubiquitin-ligase complexes known collectively as $SCF^{TIR1/AFB}$ that catalyze the ubiquitination and destruction of Aux/IAAs (Gray et al.,

2001). As mentioned above, *Aux/IAAs* belong among early response genes, thus most *Aux/IAA* genes are themselves de-repressed by the degradation of Aux/IAA proteins. This negative feedback loop provides dynamic self-control mechanism (reviewed in Kieffer et al., 2010).

During plant development numerous genes are regulated by the Aux/IAA-ARF system. For instance, in the root tip, the auxin-concentration-gradient-dependent dosage of *PLETHORA* (*PLT*) transcription factors, members of APETALA2/ethylene-responsive factors *(AP2/ERF)*, determines subsequent cell fate. High levels of PLT activity promote stem cell identity and maintenance; lower levels promote mitotic activity of stem cell daughters and further decreases required for cell differentiation (Galinha et al., 2007). Importantly, the expression of PINFORMED (PIN) auxin efflux carriers depends on PLT proteins indicating that there is a positive feedback loop promoting the root tip auxin gradient and concomitantly PLT's own expression (Aida et al., 2004; Galinha et al., 2007).

Also the initiation as well as lateral roots outgrowth relies on the auxin-driven gene expression including ARF7 and ARF19 and their putative interactor Aux/IAAs (IAA14, IAA3, IAA19 and IAA28) (Okushima et al., 2005). The LAX3 (LIKE AUXIN RESISTANT 1) auxin influx transporter expression is positively regulated by these two ARFs. Following the LAX3-dependent auxin accumulation in cells adjacent to newly formed lateral root primordium the cell wall remodeling occurs with the aid of pectate lyase and polygalacturonase to promote root proliferation (Swarup et al., 2008).

3.3. Auxin metabolism

Auxin biosynthesis, conjugation and degradation all influence the active pool of IAA, which is the most typical representative among other naturally occurring auxins, such as 4-chloro-indole-3-acetic acid, indole-3-butyric acid (IBA) and phenylacetic acid (PAA) (reviewed in Delker et al., 2008).

Most of the auxin biosynthesis is dependent on tryptophan (Trp) as a precursor, but an alternative Trp-independent pathway is postulated in *Arabidopsis*, synthesizing IAA from a Trp precursor - an intermediate in the aromatic amino acid biosynthetic pathway. Besides, these pathways can interact with each other. Each pathway seems to be spatially and temporally regulated, indicating their roles in specific developmental events (reviewed in Ljung et al., 2002). Three groups of genes code for enzymes participating in the above-mentioned pathways. These are *YUCCA* (*YUC*), *tryptophan aminotransferases TAA/TAR* and *cytochrome P450 79B2* and *79B3* (*CYP79B2, CYP79B3*). *YUC* genes in *Arabidopsis thaliana*

comprise a family of 11 members catalyzing the hydroxylation of tryptamine and display organ specific expression patterns. Multiple mutations in these genes resulted in broad developmental defects (Zhao et al., 2001; Cheng et al., 2006; Kim et al., 2007). Another group of enzymes contributing to auxin biosynthesis that converts Trp to indole-3-pyruvic acid (IPyA) are Trp aminotransferases from TAA/TAR protein family. Multiple mutants in *TAA1* and *TAR* genes showed auxin-related phenotypes such as defects in root gravitropism, embryo and cotyledon development and vasculature formation, suggesting that the *TAR* genes have a redundant function together with *TAA1* in auxin production (Stepanova et al., 2008). *CYP79B2* and CYP79B3 genes, encoding cytochrome P450s, play a key role in the production of indole-3-acetaldoxime (IAOx). This genes control IAA production in a temperature-dependent manner (Zhao et al., 2002).

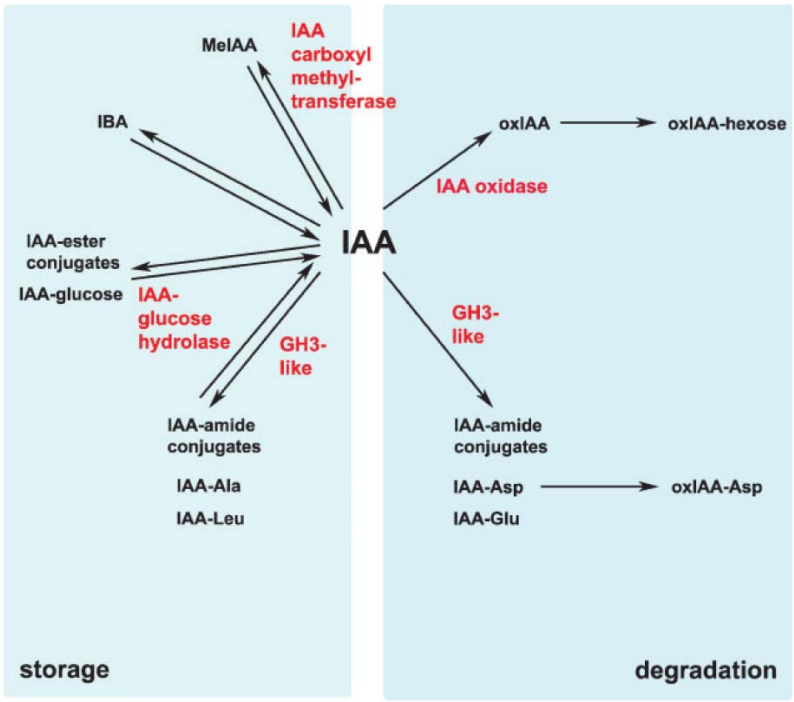

Figure 3.2: Main conjugation pathways for storage or degradation of IAA, from Chandler, (2009).

IAA conversions to other auxins, its methylation and conjugation could serve as the tool for storage or degradation. The most frequent are IAA conjugation to sugars or amino acids, its methylation, and the conversion of IAA to IBA from which free IAA can be released when needed (Bartel et al., 2001). IAA oxidases modify the indole ring and its side chains to produce oxIAA, which can be further conjugated with hexose, thus triggered to degradation (reviewed in Delker et al., 2008; Chandler, 2009).

Auxin-inducible *GH3* genes encode enzymes that conjugate IAA to amino acids for storage or to commit them to degradation (Figure 3.2). Conjugation to aspartate (Asp) or glutamate (Glu) is apparently irreversible and IAA-Asp can be further oxidized to oxIAA-Asp (Ostin et al., 1998). Auxin degradation mediated by GH3 IAA-amidosynthetases is promoted by higher auxin levels (Staswick et al., 2005). The fact that there are several GH3 enzymes that catalyze amidoconjugation to salicylic acid, abscisic acid or jasmonic acid (Staswick et al., 2002), suggests that GH3-like proteins in concert with amidohydrolases could serve as crosstalk points with other signaling pathways. Amidohydrolases release free IAA from conjugates for its immediate utilization (Bartel and Fink, 1995; LeClere et al., 2002).

3.4 Auxin transport

Cell to cell auxin transport is the major process contributing to formation of the auxin gradients governing plant development. Auxin gradients endow cells in the tissue with differential auxin concentrations which result in various physiological and developmental outcomes, depending on a specific gene expression. Inside the cells auxin in turn works as a signal for the regulation of its own transport. Therefore, the plant is able to control its development via the regulation of auxin levels and auxin flow.

In addition to long-distance auxin transport *via* the vascular system, short-distance transport occurs between cells. IAA moves between plant cells by the combination of membrane diffusion and carrier-mediated transport. Only non-dissociated form of IAA can pass through the plasma membrane by diffusion (Delbarre et al., 1996). IAA^-, a dissociated form of auxin molecule that could represent about 80% of total IAA in the apoplast (pH of 5.5) needs an influx carrier to enter the cell. Inside the cell most of the IAA molecules is in dissociated form and they can be exported out of the cell only with the aid of the auxin efflux carriers (Kramer and Bennett, 2006). Directional auxin flow occurs in vasculature transporting auxin from the shoot to the root (Goldsmith, 1977; Morris et al., 2004). Cell-to-cell carrier-mediated auxin transport triggers loading of auxin into the phloem in leaves (Marchant et al., 2002) and unloading in roots (Swarup et al., 2001). The active cell-to-cell auxin transport is also important for organ development in roots and shoots and for tropisms (Friml et al., 2002b; Friml et al., 2002a; Benkova et al., 2003). The active cell-to-cell auxin flux is provided by several membrane transporters including permeases AUXIN RESISTANT 1 (AUX1) and LIKE AUX1 (LAX) that serve as auxin influx carriers (Bennett et al., 1996; Swarup et al., 2008); PIN-FORMED (PIN) auxin efflux carriers (Gälweiler et al., 1998; Müller et al., 1998) and the multidrug resistance/p-glycoprotein (MDR/PGP) proteins from

the ATP-binding-cassette transporter family B (ABCB) (Noh et al., 2001; Verrier et al., 2008). The asymmetric localization of some of these proteins (namely PINs) underlies controlled directional polar auxin transport (PAT) (Swarup et al., 2001; Blilou et al., 2005; Geisler et al., 2005).

Proteins from PIN family play the most significant role in PAT triggering broad range of developmental events. In *Arabidopsis,* PINs are transmembrane proteins divided, according to their structure, into two subgroups, the "long" PINs (PIN1, 2, 3, 4 and 7) residing on plasma membrane (PM) and "short" PINs (PIN5, 6 and 8) residing at the endoplasmic reticulum (ER) (reviews by Křeček et al., 2009; Zažímalová et al., 2010). Long PINs can undergo constitutive recycling in membrane vesicles to and from the PM (Geldner et al., 2001) and this endocytic recycling mediates also their cellular transcytosis (Kleine-Vehn et al., 2008) needed for redirection of auxin flow required for instance for root gravitropic growth (Wisniewska et al., 2006). Interestingly, auxin itself regulates its own transport at nongenomic level by inhibiting endocytosis (Paciorek et al., 2005). Dynamic redistribution of PIN proteins to various PM domains requires clathrin-mediated endocytosis (Dhonukshe et al., 2007), which depends also on the sterol composition of the PM (Willemsen et al., 2003; Men et al., 2008). The specific sterol composition is also crucial for the interaction between PIN1 and ABCB19/PGP19/MDR1 proteins (Titapiwatanakun et al., 2009). Plant ABCB proteins, namely ABCB1 and ABCB19 function as auxin efflux carriers (Verrier et al., 2008), whereas ABCB4 seems to have both auxin influx or efflux activity depending on the auxin concentrations (Yang and Murphy, 2009). There are some examples of the co-operation between PINs and ABCB transporters during plant development (Blakeslee et al., 2007; Mravec et al., 2008; Titapiwatanakun et al., 2009). Besides PINs and ABCBs, auxin influx carriers from AUX/LAX family also contribute to the directional auxin flow. They can facilitate auxin transport also against auxin concentration gradient and - together with other auxin carriers - they operate during several developmental processes such as embryogenesis (Ugartechea-Chirino et al., 2010), hypocotyl apical hook development (Vandenbussche et al., 2010), root gravitropism (Bennett et al., 1996), lateral root development (Swarup et al., 2001), root hair development (Jones et al., 2009), phloem loading and unloading (Marchant et al., 2002), and phyllotaxis (Bainbridge et al., 2008).

3.5 Auxin binding protein 1 (ABP1)

3.5.1 ABP1, the first characterized auxin binding site

3.5.1.1 The structure of ABP1 and auxin binding characteristics

The discovery of so-called auxin binding site I was initially described in 1970s (Hertel et al., 1972; Ray, 1977) and corresponding Auxin Binding Protein 1 (ABP1) was purified from maize coleoptile membrane fractions and characterized by Löbler and Klämbt (1985a, b). In this work, ABP1 showed the highest auxin-binding affinity to synthetic auxin naphthalene-1-acetic acid (NAA), with the K_d 5.7 x 10^{-8} M at very sharp binding optimum at pH 5.5. The binding of IAA, the native auxin, to ABP1 was weaker and another synthetic auxin, 2,4-dichloro-phenoxyacetic acid (2,4-D), bound very weakly. Edgerton (1994) proposed that besides NAA, ABP1 also binds with high affinity its structural analog naphthalene-2-acetic acid (2-NAA), although it has been shown to be physiologically inactive with respect to ABP1 action (Dahlke et al., 2009).

Maize ABP1 is a small soluble glycoprotein with one glycosylation site. It occurs as homodimer with a single disulfide bond within a monomer subunit (Woo et al., 2000; Feckler et al., 2001; Woo et al., 2002). ABP1 is found predominantly within the endoplasmic reticulum, and in smaller quantities at the cell surface associated with the plasma membrane. As ABP1 has no hydrophobic regions, it is not a transmembrane protein, thus it might interact with a putative plasma membrane docking protein (Diekmann et al., 1995).

Nucleotide as well as amino acid sequence of ABP1 are highly conserved among plant species, especially in the factual auxin-binding sites labeled as boxes a, b and c (Napier and Venis, 1992; Lazarus and Macdonald, 1996). The binding pocket is hydrophobic, it contains a zinc ion coordinated by three histidines and one glutamate. Auxin binds by its carboxyl moiety to the zinc ion and the aromatic ring of auxin molecules binds three histidine residues, but the most important seems to be tryptophan 151 (Woo et al., 2002). When auxin is not bound the C-terminus of ABP1 is extended and the tryptophan 151 is pulled out of the binding site, thus representing the inactive conformation. Binding of auxin results in a small conformational change, and it stabilizes the protein structure making it more rigid; the Trp151 remains in the binding pocket (Bertosa et al., 2008). The exit of auxin from the ABP1 binding site is accompanied by protonation of auxin molecule by hydrogen-bonded water molecules and auxin then leaves the receptor towards the PM in protonated form (Bertosa et al., 2008).

3.5.1.2 Intracellular localization of ABP1

ABP1 has the N-terminal signal peptide for targeting to ER and C-terminal KDEL sequence, which serves as a signal for the retention in the lumen of ER (Hesse et al., 1989; Schwob et al., 1993). Indeed, most of the ABP1 has been found to be present in the ER and only small part seemingly might escape through the secretory system to the outer face of PM (Feldwisch et al., 1992; Jones and Herman, 1993; Bronsema et al., 1998). Diekmann et al. (1995) reported the detection of ABP1 at the surface of protoplasts released from maize coleoptiles and also showed its clustering following auxin treatment. Because of the pH optimum for the binding of auxin to the ABP1 (5.5), the PM-residing ABP1 is the first candidate to be active as an auxin receptor.

Homologs of ABP1 are ubiquitous among green plants and all of them contain the KDEL sequence, although ABP1 homologs from mosses lack this ER-retention signal, even though they are active in auxin binding (Panigrahi et al., 2009). Although the C-terminal KDEL sequence has been shown to be involved in the stability of ABP1 (David et al., 2001), the natural lack of KDEL in mosses raises the question about the importance of KDEL sequence. Moreover, it has not been shown whether auxin application would lead to the regulated release of ABP1 from the ER to PM (Tian et al., 1995; Henderson et al., 1997). Although both ABP1 mRNA and the protein have extended lifetimes (Oliver et al., 1995) it has been reported recently that ABP1 is a substrate for AtRMA2, an Arabidopis ubiquitin ligase residing in ER. This suggests that active pool of ABP1 at the ER might be controlled by proteasome (Son et al., 2010).

3.5.1.3 ABP1-mediated auxin signal transduction

As ABP1 is not a transmembrane protein and it seems to act on the outer side of PM, it should cooperate with some other protein, which serves as a docking protein and, perhaps, it even conveys the auxin signal to the next components of the signaling pathway. For long time, the search for a putative ABP1 partner was unsuccessful - until Shimomura (2006) has isolated two candidate proteins by photoaffinity crosslinking using synthetic C-terminal peptide of ABP1 from maize PMs. One of these proteins was the homologue of Arabidopsis SKU5, a GPI-anchored protein that contributes to the directional root growth processes; hence, it is a very good candidate for putative PM docking protein that can simultaneously forward auxin signal. The short α–helix on the C-terminus of ABP1 beta-barrel structure seems to be important for auxin signal transduction. Thiel et al. (1993) tested electrophysiological activity of several peptides representing various regions of ABP1 but

only the C-terminal peptide caused very rapid block of K^+ inward currents. Antibodies raised against the ABP1 C-terminus caused guard cell alkalinization and stomata closing in epidermis of orchid *Paphiopedilum tonsum* (Gehring et al., 1998). Dahlke et al. (2009) proved that auxin is retained in the binding pocket of ABP1 by boxes a and c whereas C-terminal peptide conveys auxin signal (Dahlke et al., 2009). ABP1 action is also involved in the auxin-mediated stimulation of phospholipase A2 (PLA2) isolated from soybean cells (Scherer and Andre, 1993). The products of PLA2 enzyme act as second messengers promoting H^+-ATPase activity followed by elongation of maize coleoptiles through a mechanism involving a protein kinase (Yi et al., 1996).

3.5.2 The role of ABP1 in auxin-triggered physiological processes

3.5.2.1 ABP1-mediated fast auxin responses

3.5.2.1.1 Cell expansion and elongation

Since ABP1 is present in all species from bryophytes to flowering plants (Tromas and Perrot-Rechenmann, 2010) and it is ubiquitous in all plant organs and tissues (Napier, 2001), its importance for the plant growth is obvious.

From the beginning, ABP1 was considered to mediate rapid non-genomic responses to auxin occurring on PM. Antibodies synthesized against maize ABP1 completely inhibited electrical response (the shift of transmembrane potential) to auxin in tobacco protoplasts (Barbier-Brygoo et al., 1989). This work proved that ABP1 is involved in the auxin-dependent activation of H^+-ATPase, leading to cell expansion. On the contrary, the antibodies raised against the ABP1 auxin-binding site itself caused auxin agonist activity and hyperpolarized protoplast transmembrane potential (Venis et al., 1992). Usage of auxin agonist antibodies showed that the signal for H^+-ATPase activation is initiated from outside the cell (Rück et al., 1993; Leblanc et al., 1999a). Synthetic peptides corresponding to various regions of ABP1 were tested for the auxin-like response in *Vicia* guard cells. The peptide equivalent to C-terminus was the only active one in increasing cytoplasmic alcalinization followed by enhancement of outward- and inactivation of inward-rectifying K^+ channels (Thiel et al., 1993). Tobacco plants overexpressing maize ABP1 showed higher sensitivity to lower concentrations of auxin with respect to activation of K^+ inward- or outward- rectifying channels (Bauly et al., 2000). Using surface plasmon resonance, the auxin electrical response of tobacco protoplasts was shown to involve a conformational change in *Nt*ABP1 (Leblanc et al., 1999b). In maize coleoptiles, the auxin induced growth depends on potassium currents

(Philippar et al., 1999) and the activation of inward rectifiers results in rapid cell elongation in *Arabidopsis* hypocotyls (Philippar et al., 2004).

Auxin induced H^+ extrusion by H^+-ATPase to cell wall and K^+ inward currents are followed by water uptake and turgor-driven cell wall expansion. The cell wall extension is even faster due to the low-pH-sensitive enzymes and proteins initiating cell wall loosening (reviewed in Hager, 2003).

ABP1 is required for auxin signal transduction leading to expansion of protoplast from corn coleoptiles and *Arabidopsis* hypocotyls. The antibodies raised against a part of box a, an auxin binding site, induced protoplast swelling, while antibodies raised against C-terminus inhibited this process. Synthetic C-terminal oligopeptides also caused a swelling response, indicating that the signal for the expansion is very likely conveyed by C-terminus of ABP1 (Steffens et al., 2001). Constitutive overexpession of *Arabidopsis* ABP1 in maize cell lines resulted in larger cells and inducible overproduction of AtABP1 in tobacco plants resulted in larger leaf cells but unaltered leaf size, meaning that the increased cell size was compensated by reduced frequency of cell divisions. Thus, these findings suggest the role of ABP1 in regulation of cell expansion (Jones et al., 1998). Indeed, *At*ABP1 overexpressed in tobacco leaves induced cell expansion followed by forwarding nuclei to the G2 phase of cell cycle. The highest number of ABP1 was observed in the leaf regions with lowest free IAA levels and highest rate of cell expansion. In contrast, at places of higher levels of auxin and divisions the number of ABP1 was lower. However, the antisense suppression of ABP1 in BY-2 tobacco cells led to dramatic reduction of cell expansion while the cell division was affected minimally (Chen et al., 2001a). Detailed study of cell elongation and division in BY-2 cells with antisense suppression of ABP1 revealed that cells grew as clusters of smaller cells contrasting to the linear growth creating cell files in controls. The antisense-ABP1 cells lacked auxin-induced elongation as a result of insensitivity to auxin (Chen et al., 2001b). On the other hand, the tobacco calli overexpressing maize ABP1 formed larger cells undergoing endoreduplication corresponding to two- to threefold increase in DNA synthesis (Chen et al., 2006). Interestingly the inactivation of ABP1 seems to have an inverse effect in shoot and root apices. In shoots, the leaf growth is altered due to impaired cell expansion, the cells are several times smaller (Braun et al., 2008), whereas in roots the lack of ABP1 prompts the elongation of basal meristematic cells, that are resistant to IAA (Tromas et al., 2009).

3.5.2.1.2 Cell division

Even though the action of ABP1 in regulation of cell division was suggested already in earlier studies focused primarily on cell expansion, the direct involvement of ABP1 in regulation of cell division was confirmed only recently. In tobacco suspension-grown BY-2 cells the functional inactivation of ABP1 through immunomodulation resulted in cell-cycle arrest. ABP1 has been shown to be critical for transition from gap phase 1 (G1) to phase of DNA replication (S) and from second gap phase (G2) to mitosis (M) thus controlling cell cycle (David et al., 2007).

Moreover, usage of the ABP1-antisense plants suggested the participation of ABP1 in shoot and root growth including an alteration in cell division frequency and altered pattern of endocycle induction (Braun et al., 2008; Tromas et al., 2009). The nuclei of *Arabidopsis* leaf cells lacking functional ABP1 maintained much lower content of DNA, suggesting that ABP1 is necessary to promote endoreduplication. Also in these cells the rapid decrease of early regulators of the G1/S transition, D-type CYCLIN mRNA, occurs (Braun et al., 2008). *Arabidopsis* roots inactivated in ABP1 showed rapid changes in levels of G1/S cell cycle markers. Increased accumulation of RETINOBLASTOMA-RELATED protein (RBR) and a moderate to strong decrease for cyclin dependent kinase inhibitors (KRPs) and early D-type cyclins contributed to the arrest of cell division (Tromas et al., 2009).

3.5.2.1.3 Vesicle trafficking and cell polarity

ABP1 has been shown to decrease the rate of endocytosis (Dahlke et al., 2010). This decrease might play a role in the auxin-triggered inhibition of endocytosis of some PM proteins, including the auxin efflux carriers of PIN-type, resulting in an increase of cellular auxin efflux (Robert et al., 2010). The feedback control of auxin efflux involves ABP1, which acts as an effector of clathrin recruitment to the PM, thereby promoting endocytosis of PINs (Robert et al., 2010). Auxin-dependent vesicle trafficking as well as the recycling of PINs have been shown to be regulated by Rho-related GTPases in plants (ROP). This vesicle recycling of PIN proteins consists of two subsequent parts, first the polar localization of auxin carriers is established, and than it is maintained (reviews by Xu and Scheres, 2005; Zarsky et al., 2009).

Auxin transported by PIN1 localized between two adjacent *Arabidopsis* leaf epidermal pavement cells activates Rho GTPases, ROP 2 and ROP6, which in turn promote the formation of the lob on one side and the indent on the opposite one. In other words, one cell forms the lob and the opposite cell forms the indent (Nagawa et al., 2010). The activation of

ROP2 and ROP6 occurs within 30 seconds and is ABP1-dependent as the nonfunctional ABP1 *Arabidopsis* plants don't form lobs or indents (Xu et al., 2010).

3.5.2.2 ABP1-mediated gene expression

In plants, up to now there are only two known types of auxin receptors: TIR1/AFBs and ABP1. The nucleus-localized localized TIR1/AFBs control the auxin-driven gene expression according to the developmental state.

Interestingly, the ABP1 antisense plants have been shown to have changed expression of early auxin responsive genes and the genes related to cell cycle. In dissected tobacco apices and *Arabidopsis* leaves with suppression of ABP1 activity, the levels of D-type cyclins transcripts were markedly decreased while S-phase markers histones H1 and H4 and PCNA transcripts were increased as well as the marker for M phase CYCLIN B (Braun et al., 2008).

The conditional down-regulation of ABP1 in *Arabidopsis* leaves or roots resulted in reduced auxin responsiveness followed by the decrease in transcript levels of Aux/IAA genes related to the TIR1-dependent auxin signaling pathway. Within first hour after ABP1 inactivation the decrease of IAA1, IAA5 an IAA19 (belonging to the Aux/IAAs) was recorded comparing to control in *Arabidopsis* leaves even after IAA treatment (Braun et al., 2008). In *Arabidopsis* roots the levels of transcripts IAA5, IAA6 and IAA19, belonging to the same group of Aux/IAAs as those from shoot, were decreased (Tromas et al., 2009). Consistently, heterozygous *abp1/ABP1* mutant *Arabidopsis* plants expressed IAA2, IAA11, IAA13, IAA14, IAA19 and IAA20 genes several times less than wild type after 30 min auxin application (Effendi and Scherer, 2009).

This evidence is one of the few showing the importance of ABP1 for auxin-regulated gene expression. Therefore, the ABP1, possibly standing upstream of the TIR1-dependent gene regulation and due to its PM localization, can be eligible to relay information about directional auxin flow and thus to contribute to the modulation of gene expression.

3.5.3 The role of ABP1 in plant development

3.5.3.1 Embryogenesis

Auxin is important for plant polarity establishment, which starts already in first unequal division of the zygote. The homozygous loss-of-function mutation of ABP1 results in disoriented cell division and elongation in *Arabidopsis* embryo (Chen et al., 2001b). At early stage of globular embryo, the cells of lower tier are elongating vertically. That does not occur in *abp1/abp1* embryos and the ordered flanks of cells that form by the coupling of elongation

with anticlinal divisions at later developmental stages are also lacking. The *abp1* embryo cell division abnormalities include periclinal divisions leading in disrupted polar growth and arrest in the globular stage. Alike the division order is altered in suspensor; the cells undergo additional ectopic anticlinal divisions to produce longer suspensors. These embryos are not passing on to heart stage from the globular one; neither apical-basal polarization nor lateralization occur. Thus, the *abp1/abp1* mutation is embryo-lethal (Chen et al., 2001b).

At early globular stage of embryo, PIN auxin transporters change their localization to redirect auxin flow achieving one basal auxin maximum for root primordium foundation and two apical auxin maxima for cotyledons formation (Friml et al., 2003). Embryogenic PIN gene expression is driven by MONOPTEROS (MP), a transcription factor of the auxin response factor (ARF) family, and BODENLOS (BDL), an ARF repressor and a member of the Aux/IAA protein family. Mutation in MP or BDL avoids basal embryo development and combined mutation of *mp* with NONPHYTOTROPIC HYPOCOTYL4 (NPH4), another ARF, controls both apical and basal domains creation (reviewed in Alabadi et al., 2009). Transport of auxin rather than auxin biosynthesis seems to determine the embryo patterning (Weijers et al., 2005). In addition to PINs, the ABC type-B transporters/phosphoglycoproteins (ABCBs/PGPs) contribute to establish the auxin gradients during embryogenesis and following organogenesis (Mravec et al., 2008), but the PIN proteins layout is crucial. Sauer (2007) followed PIN1 subcellular localization in *abp1/abp1* mutant embryos showing that it is non-polar. These results suggest the importance of ABP1 for embryonic development, namely for correct localization of PIN proteins.

3.5.3.2 Shoot development

Auxin plays a key role in formation of aerial organs such as leaves, flowers and secondary shoots, and it is the factor controlling phyllotaxis. Auxin has a dual role both in marking sites where new organ primordia will arise and also in maintaining their identity (Benkova et al., 2003; Alabadi et al., 2009). ABP1 is highly expressed at places of rapid growth indicating its role in meristematic development. By conditional repression of ABP1 in apical shoot meristem Braun et al. (2008) proved its importance for the leaf primordium development. ABP1 repression also resulted in changed Aux/IAA expression thus suggesting indirect relationship with the auxin-driven gene expression, dependent on $SCF^{TIR/AFB}$-mediated degradation. The *Arabidopsis* seedlings conditionally suppressing ABP1 showed dramatic reduction in growth and development; the cotyledons were small and epinastic and primary leaves delayed in emergence and did not develop further. The block of ABP1 in later

stages also led to a decrease in growth and flowering (Braun et al., 2008). Closer view to the shoot apical meristem development in tobacco revealed that the local inhibition of ABP1 in the position I1 (where leaf initiation is imminent) resulted in severe reduction of the apex and the leaf primordium initiation was significantly delayed whereas the suppression in I2 position (where leaf formation does not normally occur until after leaf formation at the I1 position) had no obvious effect. In the I1 area lacking functional ABP1 the cell size was increased and later on the division pattern became more irregular. In lower cell layers the cell expansion and division were impaired. In the primordia originated from affected I1 position, the pattern of vasculature was altered. The application of 1-naphthylphthalamic acid (NPA), the inhibitor of polar auxin transport, to I1 position led to a very similar pattern to that observed after local inactivation of ABP1 (Braun et al., 2008).

Interestingly, the authors of this extensive study (Braun et al., 2008) concluded that the phenotypic changes of *Arabidopsis* shoots caused by ABP1 inactivation were not correlated with a global modification of free IAA content. Conversely, Chen et al. (2006) revealed that cells overexpressing ABP1 had higher free auxin pool size, which cannot be explained by altered auxin transport. In cells lacking detectable ABP1, a higher rate of auxin metabolism was observed as the cells produced significantly more 1-naphthyl-1-acetyl-β-D-glucopyranose, the conjugate of NAA, a synthetic auxin which was added to the cultivation medium. Taking into account these results, apart from its auxin-signaling function ABP1 probably contributes to the establishment of proper pool of free auxin by preventing conjugation rather than altering its *de novo* synthesis.

ABP1 seems to be involved also in activity/dormancy cycle in trees, as its levels correlate with the sensitivity of cambial tissues to auxin (Hou et al., 2006).

3.5.3.3 Root development

Auxin fluxes driven by specific transporters, which are precisely distributed within cell files in the tissue, contribute to establishing and maintaining proper root organization. The transporters mediating auxin fluxes are members of PIN protein family at the first place, and ABCB transporters and auxin influx carriers AUX/LAXes. Auxin is transported acropetaly with the aid of PIN1 and PIN4, in the root cap it is redistributed laterally by PIN3 and back upward (towards the root base) by PIN2. AUX1 in addition to PINs provides directional auxin transport with auxin, facilitating auxin unloading from protophloem and backward basipetal auxin flow in the root cap (reviewed in Friml, 2003). The stem cell niche, which serves as a source of cells for root formation, is maintained due accumulated auxin in the root tip. For the

specification of the quiescent centre and stem cells are essential PLETHORA (PLT) genes coding for transcription factors, which are auxin inducible and affect also PIN genes expression. Conversely, PIN-dependent auxin gradients influence PLT transcript distribution (Aida et al., 2004).

Interestingly, ABP1 affects PLETHORA gradients and confers auxin sensitivity to root cells, thus defining the competence of the cells whether to be maintained within the meristem or to elongate. ABP1 contributes to the regulation of cell behavior at the transition zone, likely contributing to deciphering the gradient of auxin. *Arabidopsis* roots conditionally suppressing ABP1 display drastic root growth reduction, at the root apex a cell layer of columella is missing in more then 80% of roots and the differentiation of cells that have left the meristem have been forwarded. Meristematic cells above the quiescent centre have lost the capacity to divide preventing their elongation or differentiation. The area of PLTs expression was reduced to fewer cells around the quiescent centre suggesting that ABP1 activity triggers the control of transition from the meristem to the elongation zone (Tromas et al., 2009).

4 Materials and Methods

Plant material and gene constructs

BY-2 tobacco cells (*Nicotiana tabacum* L., cv. Bright Yellow 2; Nagata et al., 1992) were used for all experiments as the original reference material. Transformed BY-2 lines carrying *Arabidopsis thaliana PIN5* and *PIN7* gene under dexamethasone-inducible promoter (lines GVG-PIN5 and GVG-PIN7*)* and *GFP* intragenic translation fusion with *PIN1* gene under native promoter (line PIN1-GFP) were described previously (Petrášek et al., 2006; Mravec et al., 2009; Jelínková et al., 2010).

In this work, tobacco BY-2 cells were transformed with *Nicotiana tabacum* cDNA of *ABP1* gene driven by CaMV35S promoter in pCP60 binary vector (Li et al., 2004) in sense (*NtABP1-S*) and antisense (*NtABP1-AS*) orientation (both providing kanamycin resistance in plants) to obtain NtABP1-S and NtABP1-AS tobacco lines, respectively. These constructs were kindly provided by Catherine Perrot-Rechenmann (CNRS; Gif sur Yvette, France). GVG-AtABP1 line was obtained by the transformation of *Arabidopsis thaliana ABP1* gene under dexamethasone-inducible promoter in binary vector pTA7002 (Aoyama and Chua, 1997) providing hygromycin resistance in plants. This construct (*GVG-AtABP1*) was provided by Michael Sauer and Jiří Friml (PSB, VIB, Ghent University, Belgium).

GVG-PIN7 and GVG-PIN5 lines were retransformed by *NtABP1-S* construct to create GVG-PIN7/NtABP1-S and GVG-PIN5/NtABP1-S lines, respectively. The PIN1-GFP line was retransformed with *GVG-AtABP1* to create PIN1-GFP/GVG-AtABP1 line.

The expression of *GVG-PIN7, GVG-PIN5* and *GVG-AtABP1* genes in tobacco cells was induced by the addition of dexamethasone (DEX, 1μM) from a 30 mM stock solution in DMSO at the beginning of the sub-cultivation period. The appropriate amount of solvent was added into the controls.

Cultivation conditions, chemicals

BY-2 cells were cultivated in darkness at 27°C on an orbital incubator (Sanyo Gallenkamp, Schoeller Instruments Inc., Prague, Czech Republic; 150 rpm, 32 mm orbit) in liquid medium (3% [w/v] sucrose, 4.3 g l^{-1} Murashige and Skoog salts, 100mg l^{-1} inositol, 1mg l^{-1} thiamin, 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid, and 200 mg l^{-1} KH_2PO_4 [pH 5.8]) and sub-cultivated every 7 days. Stock BY-2 calli were maintained on media solidified with 0.6% (w/v) agar and sub-cultivated monthly. Transgenic cells and calli were maintained on

the same media supplemented with 100µg ml^{-1} kanamycin and/or 100µg ml^{-1} hygromycin and 100µg ml^{-1} cefotaxim.

All non-radiolabeled chemicals were obtained from Sigma-Aldrich (USA) or Duchefa (The Netherlands), unless stated otherwise.

[^{3}H]NAA (molar radioactivity 935 GBq.$mmol^{-1}$) was synthesized at the Isotope Laboratory, Institute of Experimental Botany, Prague, Czech Republic, [^{3}H]IAA (molar radioactivity 20 Ci.$mmol^{-1}$) was supplied by the American Radiolabeled Chemicals Inc. (http://www.arcincusa.com).

Gene transformations

The transformation protocol of An (1985) was used. A 4-ml aliquot of 3-day-old BY-2 cell suspension was co-incubated for 3 days with 100µl of an overnight culture of *Agrobacterium tumefaciens* strain GV2260 carrying appropriate plant binary vector and 20 mM acetosyringon stock in 96% ethanol was added to reach a final concentration of 20µM to facilitate transfer of T-DNA. Incubated cells were then washed three times with 150 ml of solution containing 3% (w/v) sucrose and 100 µg ml^{-1} cefotaxim and plated onto solid medium with selective antibiotics (100 µg ml^{-1} kanamycin, 100 µg ml^{-1} hygromycin and 100 µg ml^{-1} cefotaxim). Antibiotic-resistant colonies appeared after 3 to 4 weeks of incubation in darkness at 27°C. Cell suspension cultures established from these colonies were maintained as described above and appropriate antibiotics were added into the cultivation medium.

Verification of transgene presence and expression

Tobacco genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen) from transformed BY-2 cells. The tobacco *ABP1* gene fragment in lines NtABP1-S and NtABP1-AS was amplified by polymerase chain reaction (PCR) using *Taq* DNA Polymerase Kit (Fermentas). The combination of forward primer (5'-AAACTATGGGAGGTCCGGTT-3') and reverse primer (5'-AACAGGGATATGGAAGGTGC-3') produced a product of 250bp in case of transgene in the form of cDNA and 700bp product for the endogenous tobacco *ABP1*.

The expression of transgenes was performed in one-day-old constitutive lines or in one-day-old lines after induction of gene expression with dexamethasone. Total RNA was isolated using the Plant RNA Qiagen Mini-Prep and reverse transcription polymerase chain reaction (RT-PCR) was performed using Qiagen® OneStep RT-PCR according to the manufacturer's manual. The *Arabidopsis PIN7* gene fragment was amplified using forward primer (5'-TCGTTGTTCTTCAGTGTAT-3') and reverse primer (5'-CTCCACACCATTATCAAT-3')

giving a product of 1000bp. The *NtABP1* gene fragment was amplified using the same primers like in PCR from genomic DNA. *AtABP1* gene fragment was amplified using forward primer (5'-TAGTCGACAAAATGATCGTACTTTCTGT-3') and reverse primer (5'-CTGAATTCTAGATTAAAGCTCGTCTTTTTGTGATTC-3') giving a product of 500bp. *AtPIN5* gene fragment was amplified using forward primer (5'-CCGCTCGAGGAAGCTTTGTTTTCTTTCCTTTCGT-3') and reverse primer (5'-AAGAAGATCCTCCTCAGAAT-3') giving a product of 1200bp.

Microscopic determinations of cell growth parameters, fluorescence microscopy

For the determination of cellular dimensions, Nomarski DIC microscopy was performed using Nikon Eclipse E600 microscope (Nikon, Japan) and images were grabbed with color digital camera (DVC 1310C, USA). Using LUCIA image analysis software (Laboratory Imaging, Prague, Czech Republic) diameter and length of 200 cells of every sample were interactively measured and expressed as the reciprocal plot reflecting the distribution of cellular dimensions in the sample. In addition, categorized length distribution of cells from 10 optical fields of every sample was presented as the percentage of total cell number (approximately 200 cells). Every category corresponds to the cell length interval defined on the x axis by its maximum (intervals 0-30, 30-50, 50-70, 70-100, 100-150, 150-220 μm). Number of individual cells per cell file was determined from cells of 10 optical fields and expressed as the percentage of total cell number (approximately 400). Cell density was determined by counting cells using Fuchs–Rosenthal haemocytometer slide during whole cultivation cycle, Individual values represent the average of at least four aliquots of every sample. Stock solutions of NPA (100 mM) or PBA (100 μM) in DMSO were added to the cultivation medium at the beginning of subcultivation period to reach a final concentration of 10μM for NPA or 10nM for PBA.

Immunofluorescently labeled cells were observed using epifluorescence microscope Nikon Eclipse E600 (Nikon, Japan) equipped with appropriate filter sets for Alexa 555 and color digital camera (DVC 1310C, USA).

Quantification of cell viability

Cell viability was assessed with fluorescein diacetate (FDA) staining living cells and propidium iodide (PI) staining dead cells. 40 μl of FDA solution in cultivation medium (40 μl of stock solution in acetone (2 mg ml^{-1}) diluted with 15 ml of cultivation medium) and 4 μl of PI stock solution (1 mg ml^{-1} in water) were mixed with 40 μl of three-day-old BY-2 cells

(cultivated in the media supplied with 1μM 2,4-D or 1μM IAA) directly on microscope slide. Using an epifluorescence microscope (Nikon Eclipse E600) equipped with appropriate filter sets, DIC optics, color digital camera (DVC 1310C, USA) and LUCIA image analysis software (Laboratory Imaging, Prague, Czech Republic) the viability was determined from at least 10 optical fields on each of three separate slides as a percentage of fluorescing cells (at least 400 cells were counted in each sample).

Fluorescence recovery after photo-bleaching (FRAP)

Zeiss LSM 5 Duo confocal microscope (Carl Zeiss, Jena, Germany) with appropriate filter sets for GFP detection (excitation 488 nm, emission 505-550 nm) and 40x C-Apochromat water immersion objective (NA=1.2) were used. For bleaching experiments, the region of interest (ROI) of defined size (a rectangle of 40x20 pixels with the membrane in the centre) was interactively applied at the transversal plasma membranes of BY-2 cell files. Bleaching with maximal laser intensity was followed by 160 seconds tracking of fluorescence recovery with imaging every 7 seconds. For the compensation of nonspecific changes, another rectangle ROI (100x20pixels) was applied using Carl Zeiss Image examiner software on non-bleached part of transversal plasma membrane. In this ROI, the fluorescence was measured and values were corrected for this background. The resulting values are the mean of at least 8 measured cells and reflect the rate of the fluorescence recovery (how much relative fluorescence is recovering). Three-day-old cells induced by 1μM dexamethasone in DMSO at the beginning of subcultivation were used for all FRAP experiments. Cells were pre-treated with 20μM BFA (added from 20 mM stock solution in DMSO) for 30 minutes and with 5μM NAA (added from 10mM stock solution in 96% ethanol) for 60 minutes before the FRAP experiment.

Production of recombinant ABP1 and antibody production

The cDNA sequence of NtABP1 inserted into pQE3xHis (Qiagen) was expressed in *Escherichia coli* XL1 blue strain. The construct was kindly provided by Karel Wolf (IEB ASCR, Prague, Czech Republic). Bacteria from the single colony were cultivated in 2 ml of LB medium (5 g l^{-1} select yeast extract, 10 g l^{-1} pepton, 10 g l^{-1} NaCl; pH 7.5) supplemented with 100 μg ml^{-1} ampicilin over night at 37°C. From this a 0.5 ml aliquot was added to another 2 ml of LB medium and cultivated for 1 h at 37°C. After the induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) 1 ml of culture was inoculated to 1 l of LB medium and cultivated for 4 hours. Cells were centrifuged at 5000 *g*, pellet re-suspended in

100 ml H_2O and centrifuged at 5000 *g*. About 2 g of pellet was obtained and re-suspended in 10 ml of extraction buffer (8 M urea, 100 mM $NaH_2PO_4.2H_2O$, 10 mM Tris Base, pH 8), gently mixed for 1h and centrifuged at 5000 *g* at RT for 20 min. Supernatant was mixed with 2 ml of Ni-NTA resin (Qiagen) and loaded to a column (the solution flow 0.5 ml/min). The protein bound on the resin was eluted by the extraction buffer in range of pH 5.9 - 4.5. Eluted protein in extraction buffer was transferred to phosphate buffered saline (PBS; 8 g l^{-1} NaCl, 0.20 g l^{-1} KCl, 1.44 g l^{-1} Na_2HPO_4, 0.24 g l^{-1} KH_2PO_4) using two-step dialysis. Dialysis purified ABP1 protein (4.5 mg ml^{-1}) in sterile PBS buffer was used for the immunization of one rabbit (Institute of Physiology AS CR, v.v.i.) in two subsequent injections after two weeks. The serum containing the antibody was obtained after one month.

Immunocytochemistry

Modified indirect immunofluorescence method described in Petrášek et al. (2003) was used for the visualization of ABP1 in tobacco cells. Cells in exponential growth phase were adhered onto the microscopical slides coated with 0.1% polyethyleneimine. These slides were attached to a spacer on another microscope slide that allows diffusion of washing solutions in between two slides. Samples were placed into the humid chamber of slide module of InSituProVSi (Intavis, Germany) system for automated immunocytochemistry. Cells were pre-fixed 20 min with 100 μM MBS and 40 min with 3.7% (w/v) PFA in buffer consisting of 50 mM PIPES, 2 mM EGTA, 2 mM MgSO4, 1% Triton X-100 (w/v) pH 6.9, at 25°C. After three-times 10 minutes wash in stabilizing buffer (SB) (50 mM PIPES, 5 mM EGTA, 2.5 mM $MgSO_4$) cells were treated for 7 min with the enzyme solution (1% (w/v) macerozyme and 0.1% (w/v) pectolyase) at 25°C followed by another three-times 10 minutes wash in SB. After treatment with with 0.5% (w/v) bovine serum albumin in PBS for 30 min, cells were incubated for 1 h at 37°C with the anti-NtABP1 rabbit serum primary antibody. After three-times 10 minutes washing in PBS the secondary antibody was applied for 1 h at 37°C. Ultrapure water was used for final repeated wash. The cells on the microscope slides were mounted into Mowiol (Polysciences) solution, observed immediately and stored at 4°C.

Anti-NtABP1 polyclonal rabbit serum antibody was used as primary antibody at 1:500 (in PBSA); secondary anti-rabbit antibody (Alexa 555, Molecular Probes) was used at 1:1000 (in PBSA). Tobacco ABP1-overexpressing cells were stained after two-day cultivation in media containing 1 μM 2,4-D or 1 μM IAA. Auxins were added at the beginning of subcultivation cycle.

Protein extraction and western blotting

0.6 g of three-day-old cells were homogenized in 600 µl of extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitors cocktail Sigma (P9599), 1 µl/100 µl; pH 8.0) with an acid-purified sand (Sigma-Aldrich) using pestle and mortar. Total protein fraction in supernatant was obtained after centrifugation at 800*g* at 4°C for 5 min.

Sodium dodecyl sulfate acrylamid gel electrophoresis (SDS-PAGE) was used for the separation of proteins from total fraction. 500 µl of this fraction was mixed with 100µl of loading buffer (7 ml 0.5 M Tris-HCl buffer (pH 8.8) 3.6 ml glycerin, 1 g sodium dodecyl sulfate (SDS), bromphenol blue), boiled for 5 min, centrifuged at 5000 g at 4°C for 5 min and loaded onto SDS-PAGE gel. Proteins were separated for 1.5h at 180V (Biometra); equal amounts of protein (about 100 µg) in were loaded. The protein concentration of the samples was checked using a spectrophotometer set at a wavelength of 750 nm and a Lowry assay (Bio-Rad RC DC Protein Assay, 500-0117).

Proteins from electrophoresis were transferred onto polyvinylidene difluoride (PVDF) membrane using electroblotting for 1.5h at 350mA (Biometra). PVDF membrane with proteins was blocked from unspecific antibody binding overnight in Tris-buffered saline (TBS; 20 mM Tris base, 150 mM NaCl, 1% Triton, pH 7.4) with 2% (w/v) low-fat milk. Membrane was incubated with primary rabbit polyclonal anti-ABP1 antibody (1:100) in TBS for 1h at RT. After wash in TBS, membranes were incubated with secondary HRP-conjugated anti-rabbit antibody (1:2000; Sevapharma, Czech Republic) in TBS for 1h at RT. ECL detection kit (Amersham Biosciences, U.K.) was used for the vizualization of signal. Membranes treated with ECL reagents were placed in the cassette containing RTG film, which was then developed and scanned using Epson V700 scanner.

Auxin accumulation assays

As described in Petrášek et al. (2003; 2006) auxin accumulation was measured in suspension-cultured tobacco cells 24 h after the subcultivation unless stated otherwise. Cultivation medium was removed using filtration through nylon cloth (20 µm mesh) under reduced pressure, cells were re-suspended in the uptake buffer (20 mM MES, 10 mM sucrose, 0.5 mM $CaSO_4$, pH adjusted to 5.7 with KOH) and equilibrated for 45 min on the orbital shaker at 27°C. Then, cells were collected by filtration, re-suspended in the fresh uptake buffer and incubated for 1.5 h under the same conditions. The density of suspension was adjusted to about $6x10^5$ cells/ml. At the beginning of the accumulation assay [^{3}H]NAA (as a

good substrate for auxin efflux carriers in tobacco cells; Delbarre et al. 1996; Petrášek and Zažímalová 2006) was added to the equilibrated cell suspension to give a final concentration of 2 nM. The accumulation of labeled auxin was measured at certain time points within 25 min, 0.5 ml aliquots of suspension were withdrawn and accumulation of label in the cells was terminated by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. The cells embedded on filters were transferred to scintillation vials, extracted in ethanol for 30 min, and radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 2900TR scintillation counter, Packard Instrument Co., Meriden, CT, USA) with automatic correction for quenching. All measurements were done at least in triplicates and standard errors of the mean are shown. Values are expressed as pmols of the particular auxin accumulated per million cells or as percentage of control in the time 20 min after addition of labeled auxin. The inhibitors in final concentrations of 10μM for NPA and 20μM for BFA (added from 20 mM stock solution in DMSO) or 50μM for Tyrphostin A23 (added from 50 mM stock solution in ethanol) were applied at the time zero of each particular experiment.

Phospholipase activity determinations

The activity of phospholipases PLA2, PLD and PC-PLC was measured *in situ* with the fluorescent substrate Bodipy-phosphatidylcholine (BODIPY-PC; Invitrogen, Czech Republic, D-3771) as described in (Pejchar et al., 2010). To three-day-old BY-2 cells in suspension (0.056 g cells/ml of cultivation medium) the BODIPY-PC fluorescent substrate was added (0.66 μg/ml of suspension). After 2 h incubation on the orbital shaker at 26.5°C and 130 rpm the reaction was stopped using 4 ml cold solution of methanol and chloroform (2:1; v/v). After the 30 min extraction in darkness at room temperature, 2ml of 0.1 M KCl was added and incubated in darkness at 4°C for another 30 min. Samples were centrifuged at 420 g at 4°C for 15 min to separate water and organic phase. 950 μl of the lower phase were evaporated to dryness by a vacuum evaporator, re-dissolved in 50 μl of ethanol and after 10 min used for high performance thin-layer chromatography (HP-TLC). The products of the enzymatic digestion (Bodipy-diacylglycerol, BODIPY-DAG; Bodipy-phosphatidic acid, BODIPY-PA; Bodipy-lysophosphatidyl choline, BODIPY-LPC) were separated by HP-TLC and analyzed using fluorescence scanner Fuji FLA-7000 (Fujifilm, Japan) and Multi Gauge (Fujifilm, Japan) software. Values expressed as the percentage of control BY-2 cells represent the mean of two measured samples.

Cells used for this experiment were cultivated in standard medium. To wash 2,4-D out from cells, before the addition of BODIPY-PC cells were equilibrated using the same procedure as for the auxin accumulation assays.

IAA metabolic profiling

The method described in (Mravec et al., 2009) was used. Twenty-four hours after subcultivation, cells were prepared for the experiment by equilibration in uptake buffer following the same protocol as before the auxin accumulation assays. Cells were incubated with 20 nM [^{3}H]IAA (specific activity 20 Ci mmol, American Radiolabeled Chemicals) for a period of 2.5 h. Extraction and purification of auxin metabolites were performed as described (Dobrev and Kaminek, 2002). Briefly, 200 mg of BY-2 cells were placed in eppendorf tubes, 1.5 ml of cold Bieleski's solution was added (methanol/water/formic acid, 15:4:1, v/v/v) and cells homogenized with Retsch homogenizator at 30 Hz for 4 min. After the overnight incubation at -20°C samples were centrifuged at 18000 rpm at 4°C (Jouan AM2.18, France). Supernatant was collected in falcon tubes and pellet was again re-suspended in 1 ml of cold Bieleski's solution, homogenized and centrifuged. Supernatant was added to the previous one and evaporated to dryness. Dry fraction was diluted in 5 ml of 1 M formic acid and purified on an Oasis MCX mixed mode, cation-exchange, reverse-phase column (Waters, 6 cc/150 mg). IAA metabolites were eluted from MCX columns by 5 ml of methanol and evaporated to dry. Dry samples were dissolved in 20 µl of 50% (v/v) acetonitrile, diluted by the addition of 80µl distilled water and used for the detection of [^{3}H]IAA metabolites by high performance liquid chromatography (HPLC). The column eluate was monitored by a Ramona 2000 flow-through radioactivity detector (Raytest) after online mixing with three volumes of liquid scintillation cocktail (Flo-Scint III, Packard BioScience Co.). The radioactive metabolites of [^{3}H]IAA were identified on the basis of comparison of their retention times with authentic standards. The plots show representative profiles of [^{3}H]IAA in overlay of three repetitions for each cell line.

5 Results

5.1 Auxin binding protein 1 mediates both cell division and expansion in tobacco BY-2 cells

As described in the introduction (chapter 3.5.2), there is multiple evidence on the role of ABP1 in the processes of cell division and cell expansion mediated by auxin. Various experimental systems including embryos, leaves, roots, cell suspension cells and protoplast were used to demonstrate the activity of ABP1 in these processes.

In this work, tobacco BY-2 cells (Nagata et al., 2002) were used to address the impact of over-expressed or down-regulated *ABP1* gene on the cell phenotype and cell division and growth parameters. Tobacco BY-2 cells were transformed with cDNA of tobacco *ABP1* under constitutive 35S promoter (NtABP1-S cells) and cDNA of Arabidopsis *ABP1* under inducible promoter (GVG-AtABP1 cells) and the presence and expression of corresponding transgene was verified using PCR or RT-PCR (Figure 5.1) using specific primers (see Materials and Methods).

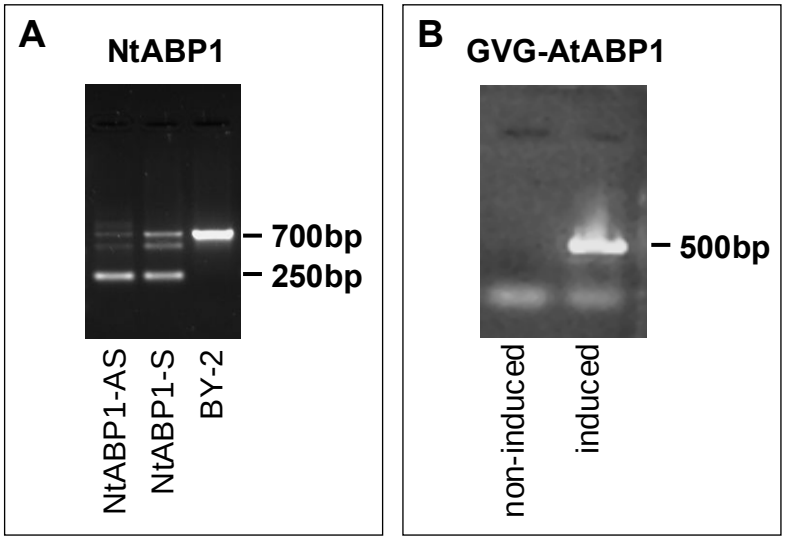

Figure 5.1: Verification of transgene presence and expression in one-day-old NtABP1-S, NtABP1-AS and GVG-AtABP1 cells. **(A)** *NtABP1-S* and *NtABP1-AS* presence confirmed by PCR (700bp fragment of endogenous gene, 250bp fragment of transgenic cDNA). **(B)** Inducible *AtABP1* expression in GVG-AtABP1 cells verified by RT-PCR, cells were induced with 1 μM dexamethasone for 24 hours (500 bp fragment).

When cultured in standard MS medium containing 1μM 2,4-dichlorophenoxyacetic acid (2,4-D), NtABP1-S cells (Figure 5.2A) and induced GVG-AtABP1 cells (data not shown) showed similar phenotype of cell chains in the exponential growth phase. Similarly, both growth rates expressed as number of cells/ml (Figure 5.2B) and cell length categories (Figure

5.2C) were comparable in NtABP1-S and control BY-2 cells. Inducible overexpression of *AtABP1* did not alter the distribution of cell files categories (Figure 5.2D) indicating that there were not ABP1-induced changes in the population of cells growing in standard concentration of 2,4-D (1μM).

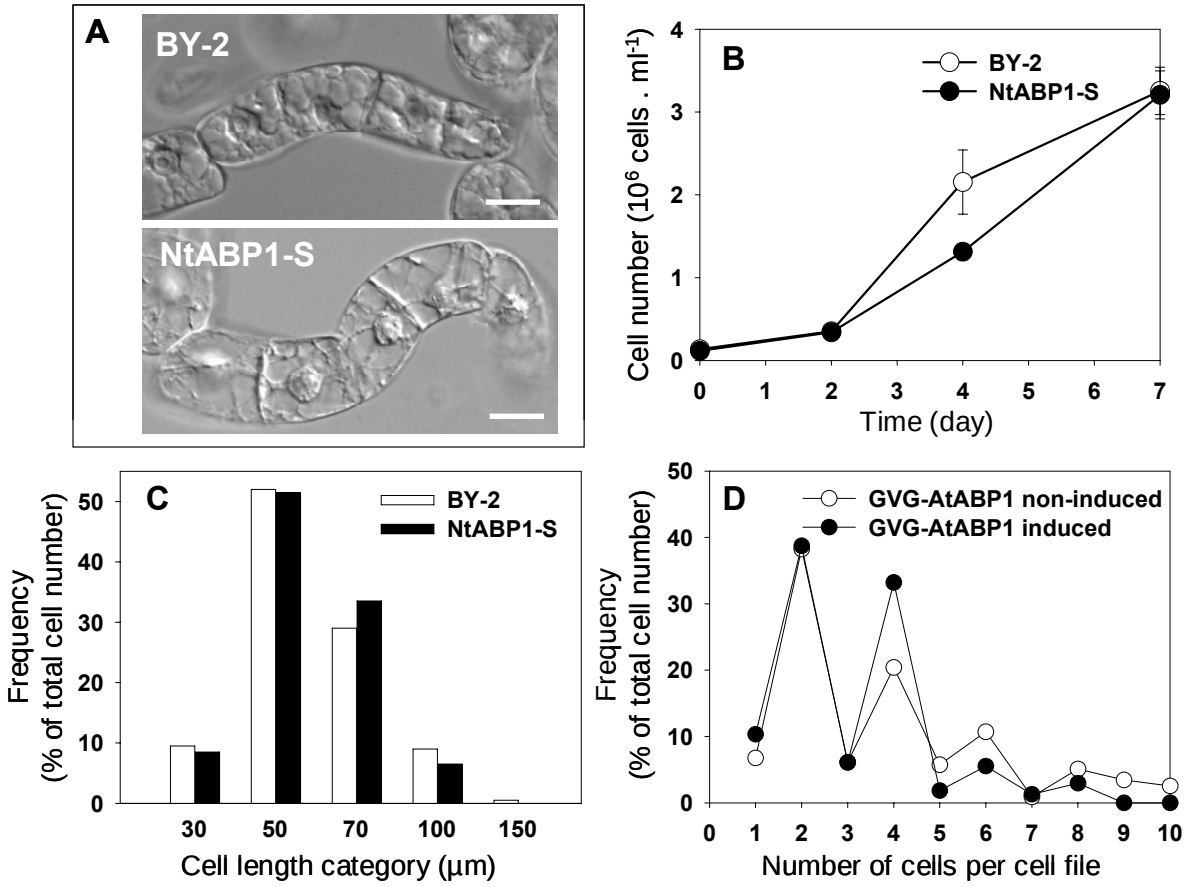

Figure 5.2: Cell growth parameters of tobacco BY-2 cells over-rexpressing constitutively tobacco ABP1 (NtABP1-S) or Arabidopsis AtABP1 under dexamethasone-inducible promoter (GVG-AtABP1). **(A)** Nomarski DIC images of two-day-old tobacco cells BY-2 and NtABP1-S cells. Scale bars 20μm. **(B)** Growth curves of tobacco cell line BY-2 and NtABP1-S cell line. Error bars SEM (n=4). **(C)** Categorized cell length distribution of two-day-old BY-2 cells and NtABP1-S cells. **(D)** The incidence of the cell chains categorized according to the number of cells per chain in three-day-old GVG-AtABP1 cells induced by 1μM dexamethasone, the solvent (DMSO) was added to non-induced cells.

To test whether ABP1 would mediate the response to decreased or increased levels of 2,4-D in the culture medium, dimensions of cells cultured three days in both 10-times decreased concentration of 0.1 μM 2,4-D and 5-times increased concentrations of 2,4-D (5 μM) were determined in the inducible GVG-AtABP1 cells. The inoculum contained preferentially two-cell files and individual cells. There was no difference in the response of GVG-AtABP1 non-induced and induced cells to 0.1 2,4-D (Figure 5.3A, B), which was

characterized by the inhibition of cell division and accelerated cell elongation in comparison with control (1 μm 2,4-D). In contrast, while 5 μM 2,4-D slightly increased the cell division activity in non-induced cells and thus the incidence of small cells, induced cells did not show this response at all, having the same phenotype as in the control 1 μM 2,4-D.

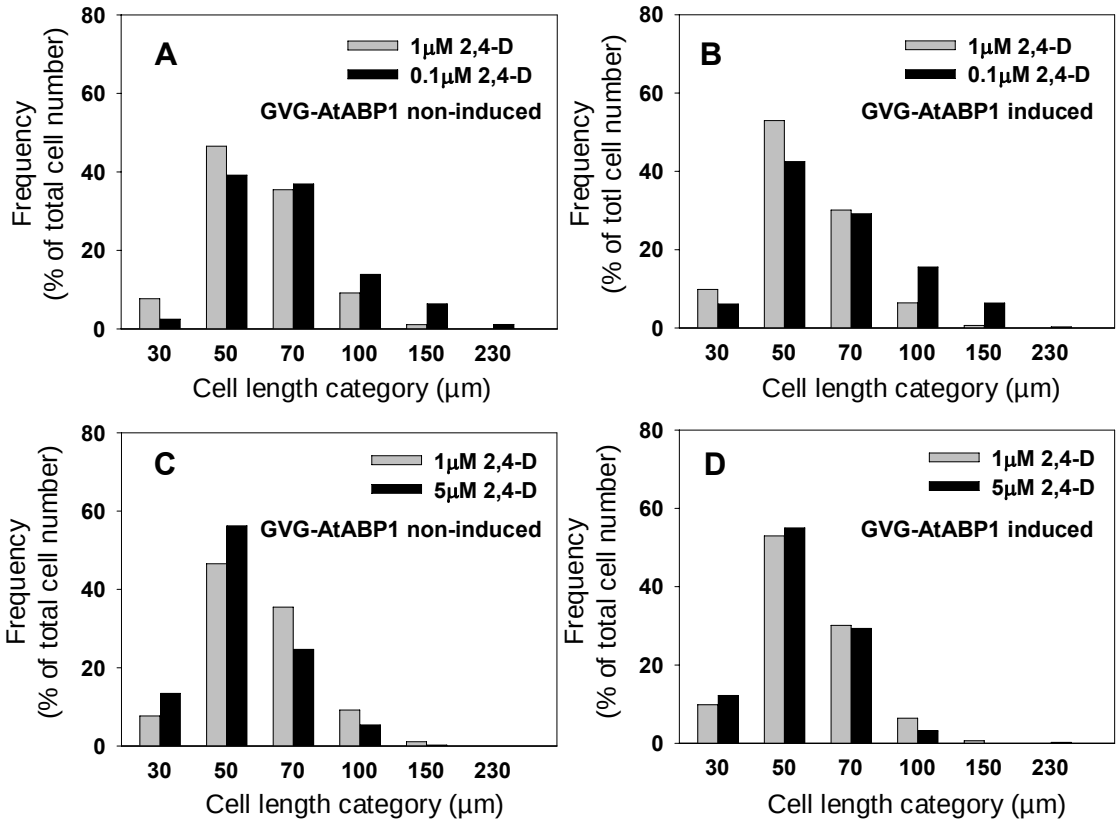

Figure 5.3: Categorized cell length distribution of three-day-old BY-2 **(A, C)** and GVG-AtABP1 **(B, D)** cells cultured in 0.1 μM or 5 μM 2,4-D, in comparison with control 1 μM 2,4-D.

To further address the effect of ABP1 in the cell expansion and cell division BY-2 cells were transformed with cDNA of tobacco *ABP1* in antisense orientation under constitutive promoter (NtABP1-AS cells) and the expression of corresponding transgene was verified using PCR (Figure 5.1A). During exponential phase of growth, cells of NtABP1-AS line formed cell files with higher number of cells in comparison with NtABP1-S cells; cells within these files were extremely short (Figure 5.4A) and cell files often formed clusters indicating the absence of cell elongation. This finding is much clearer after 7 days of cultivation, when NtABP1-S cells are already in the stationary phase of growth characterized by intensive elongation, while NtABP1-AS cells did not elongate at all (Figure 5.4B). Whereas cells of NtABP1-S line undergo at least four division cycles, NtABP1-AS cells divide not more than

once during seven-day-cultivation (Figure 5.4C). The reason why there are long cell files present is that they are subcultured from inoculum already containing cell files.

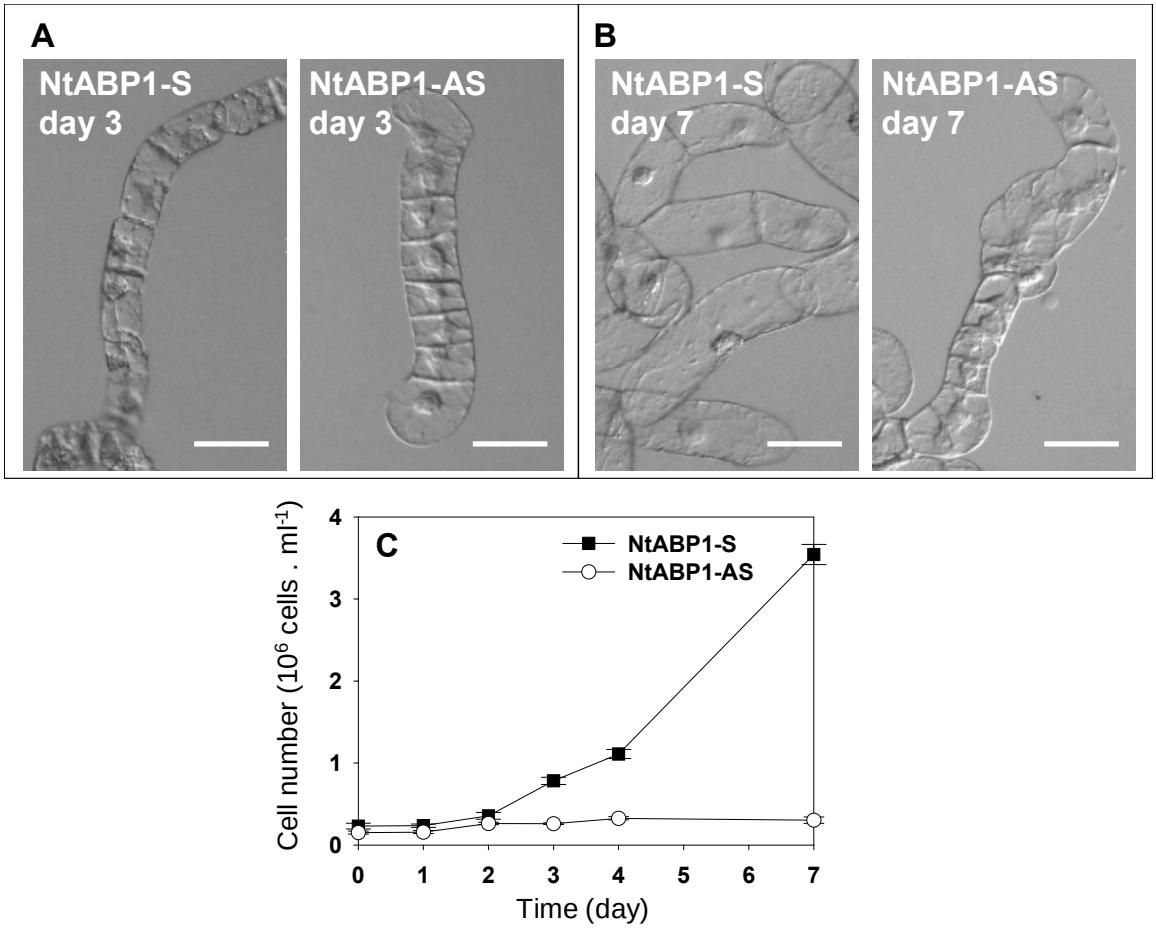

Figure 5.4: Cell growth parameters of tobacco BY-2 cells over-expressing constitutively tobacco antisense ABP1 (NtABP1-AS), the comparison with NtABP1-S. **(A)** Nomarski DIC images of three-day-old NtABP1S and NtABP1-AS cells. **(B)** Nomarski DIC images of seven-day-old NtABP1S and NtABP1-AS cells. Scale bars, 50µm. **(C)** Growth curves of tobacco cell lines NtABP1-S and NtABP1-AS. Error bars SEM (n=6).

To search for the localization of ABP1 during cell division and cell expansion, polyclonal antibody against tobacco ABP1 has been raised in rabbit (see chapter 4, Materials and Methods) and its specificity tested with immunoblot (Figure 5.5D). Using this antibody, it was shown that in BY-2 cells cultured in standard 2,4-D medium the ABP1 protein was localized exclusively at the endoplasmic reticulum (Figure 5.5A) and plasma membrane was void of any signal (arrows, Figure 5.5A). In contrast, when 2,4-D was replaced with 1 µM IAA and cells cultured three days, the signal was observed at the plasma membrane between individual cells (Fig. 5.5B). Both BY-2 and NtABP1-S cells cultivated in IAA were dividing much less than control and individual cells elongated significantly (Figure 5.5C).

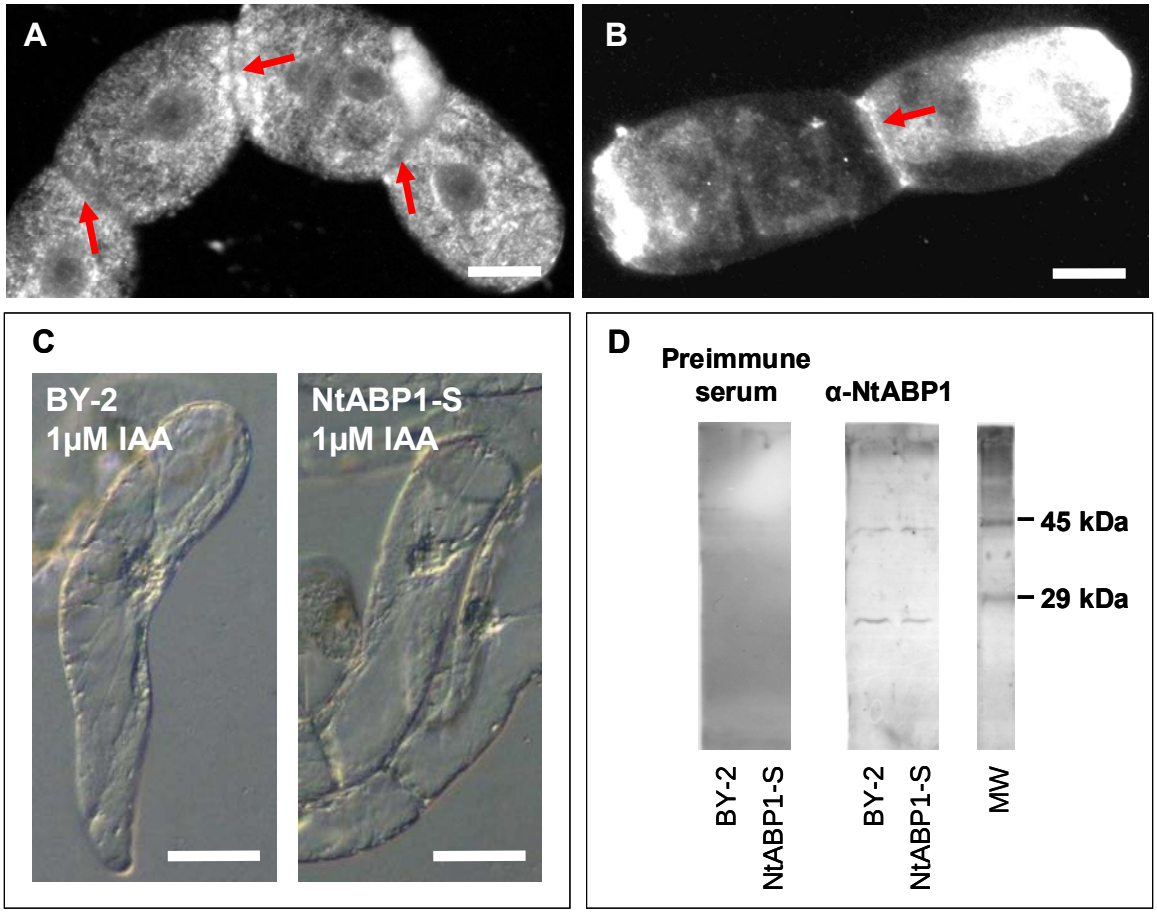

Figure 5.5: Indirect immunofluorescence and immunoblot staining of NtABP1 with rabbit polyclonal α-NtABP1 antibody. **(A, B)** Immunostaining of NtABP1 in three-day-old BY-2 cells cultured in standard medium supplemented with 1µM 2,4-D **(A)** or 1µM IAA **(B)**. Secondary α-rabbit Alexa 555 antibody. Scale bars 20µm. **(C)** Nomarski DIC images of three-day-old cells treated with 1µM IAA, scale bars 50µ. **(D)** Immunoblot with preimmune serum and polyclonal α-NtABP1 antibody. Total protein extracts from three-day-old NtABP1-S BY-2 cells.

These results suggest that the preferential role of ABP1 localized at the plasma membrane is to mediate cell expansion/elongation. For the processes of cell division, endoplasmic-reticulum-localized pool of ABP1 is important.

5.2 ABP1 mediates intercellular auxin transport

Based on the experiments described above, it could be speculated that ABP1 is involved in the regulation of transport of auxin from cell to cell. Therefore, the growth response of NtABP1-S and GVG-AtABP1 cells to the application of the inhibitor of active auxin efflux 1-naphthylphthalamic acid (NPA) was studied. The application of 10 µM NPA for three days to the control BY-2 cells resulted in the higher amount of shorter cells in cell files in comparison

with control (Figure 5.6A). In contrast, in NtABP1-S cells the cell elongation was stimulated (Figure 5.6B) suggesting the interference of ABP1 with the activity and/or localization of auxin efflux carriers.

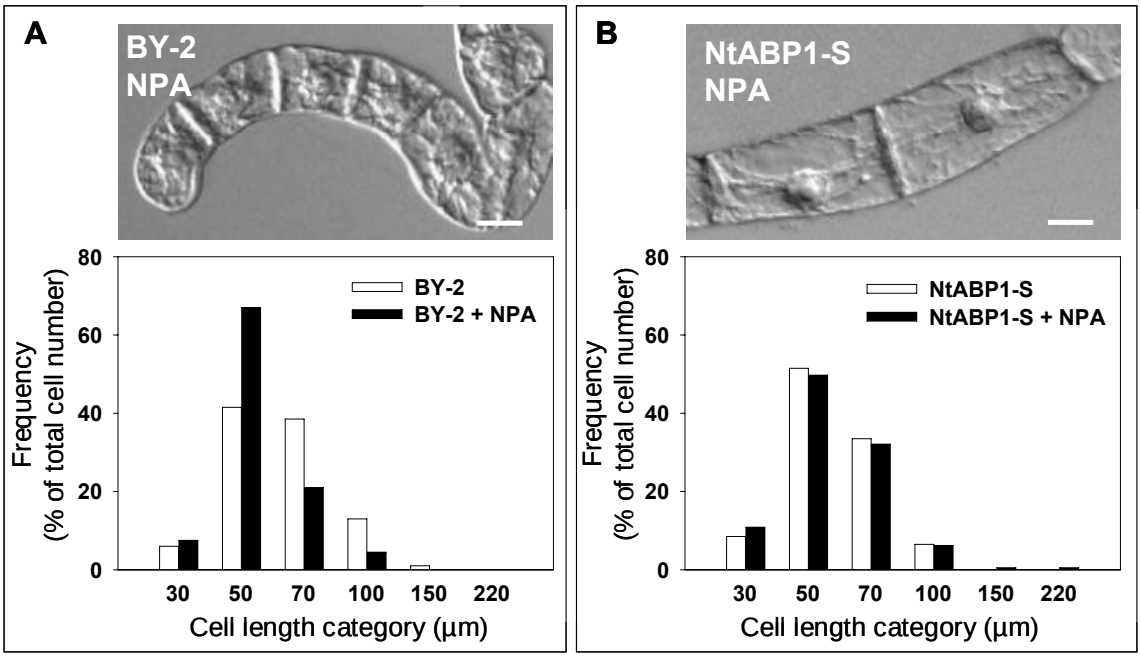

Figure 5.6: Cell growth parameters of tobacco BY-2 cells **(A)** and NtABP1-S **(B)** cells treated for three days with 10µM NPA. Nomarski DIC images, scale bars 20µm (upper panels) and categorized cell length distribution (lower panels).

Measurement of the accumulation of radioactively labeled NAA (reflecting activity of the auxin efflux machinery) showed that there is only very weak stimulatory effect of the over-expressed GVG-AtABP1 on the active auxin efflux (Figure 5.7A). However, as measured using NtABP1-S cells, the NPA-sensitive auxin efflux was decreased compared to control cells, pointing again to the interference of ABP1 with the activity and/or the localization of the auxin efflux carriers. Importantly, the auxin efflux assay was performed in one-day-old cells (Figure 5.7B) to detect possible early ABP1-induced changes on auxin efflux, as they could possibly be the reason of phenotypic changes observed after three days.

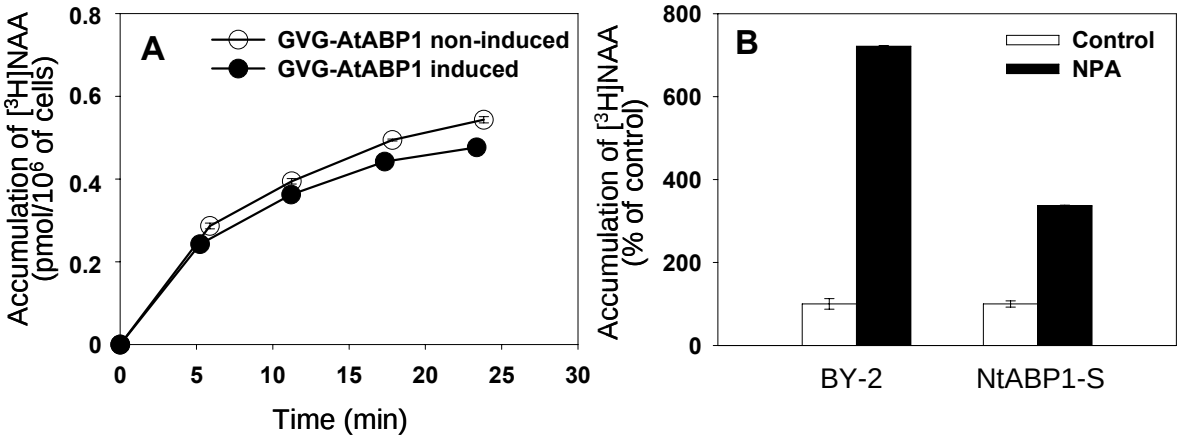

Figure 5.7: [^{3}H]NAA accumulation reflecting the activity of the active auxin efflux. **(A)** Kinetics of [^{3}H]NAA accumulation in 2-day-old GVG-AtABP1 cells. The expression of AtABP1 was induced by 1μM dexamethasone and DMSO as solvent was added into control cell suspension. **(B)** [^{3}H]NAA accumulation in one-day-old BY-2 and NtABP1-S cells treated with 10μM NPA at the time 20 min after addition of labeled NAA together with NPA. Data expressed as the percentage (corresponding untreated control=100%). Error bars show SEM (n=4).

Interestingly, in contrast to treatments with NPA, the application of another auxin transport inhibitor 2-(1-pyrenoyl) benzoic acid (PBA) did not induce any changes in cell size of control BY-2 (Figure 5.8A) and NtABP1-S cells (Figure 5.8B). Therefore, the ABP1-mediated control of auxin transport does not seem to be related primarily to actin dynamics which was previously reported to be influenced by PBA (see Introduction and Discussion).

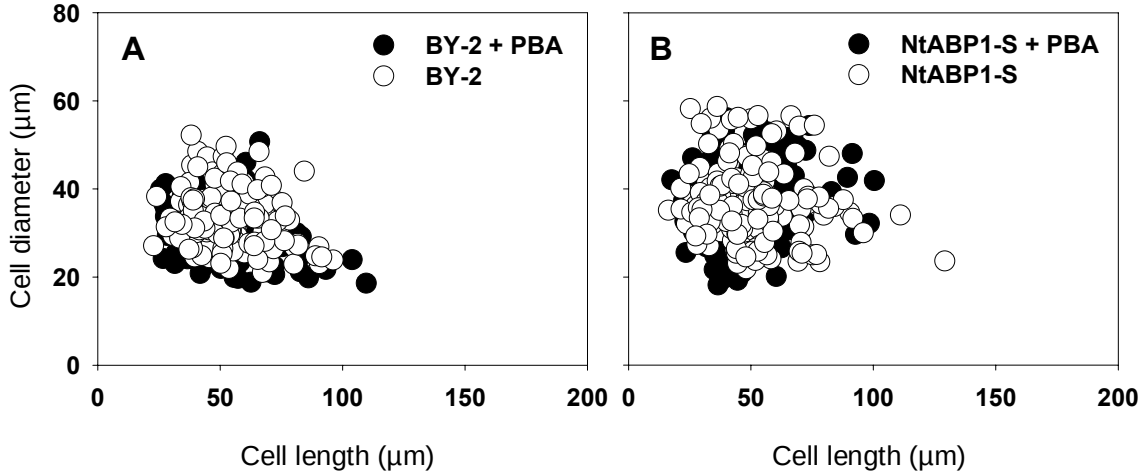

Figure 5.8: Reciprocal plot of cell dimensions of three-day-old BY-2 **(A)** and NtABP1-S **(B)** cells treated with 10 nM PBA, DMSO as solvent was added into control.

The results with NPA have suggested the involvement of ABP1 in auxin transport, namely in the active cellular auxin efflux. Since PIN-dependent auxin efflux seems to be more

sensitive to NPA compared to other auxin exporters (Petrášek et al., 2006),the effect of ABP1 on the cell-to-cell transport was further studied in tobacco BY-2 cells double-transformed with tobacco *ABP1* under 35S constitutive promoter and dexamethasone-inducible Arabidopsis *PIN7* (GVG-PIN7). The alternative double-transformed ABP1/PIN line allowing *in vivo* observation of PIN1 was prepared by the transformation with Arabidopsis *ABP1* under dexamethasone-inducible promoter and constitutive *Arabidopsis PIN1::PIN1-GFP* intragenic fluorescence protein translational fusion construct. Single tobacco lines GVG-PIN7 and PIN1-GFP were described (see Materials and Methods). Resulting double-transformed lines were verified with RT-PCR (Figure 5.9) and designated GVG-PIN7/NtABP1-S and PIN1-GFP/GVG-AtABP1. The objective was to address the role of ABP1 in cells, where auxin transport through PIN proteins is enhanced.

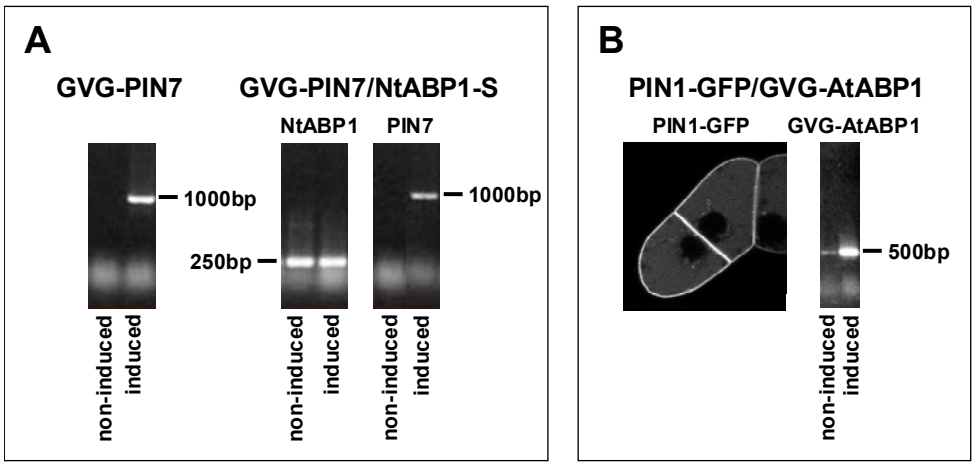

Figure 5.9: RT-PCR verification of transgene expression in GVG-PIN7, GVG-PIN7/NtABP1-S and PIN1-GFP/GVG-AtABP1 cells induced with 1 μM dexamethasone for 24 hours. **(A)** Inducible *PIN7* expression in GVG-PIN7 cells (1000 bp fragment) and *PIN7* (1000 bp fragment) at the background of constitutive *NtABP1* expression (250 bp fragment) in GVG-PIN7/NtABP1-S cells. **(B)** Inducible *AtABP1* expression (500 bp fragment) and PIN1-GFP fluorescence in three-day-old PIN1-GFP/GVG-AtABP1 cells induced with 1 μM dexamethasone.

Upon induction with dexamethasone GVG-PIN7 cells showed enhanced cell elongation and decreased cell division activity (Figure 5.10A). In contrast, the induction of *PIN7* expression in GVG-PIN7/NtABP1-S cells did not promote any cell elongation and also cell division activity was not decreased (Figure 5.10B). This indirectly suggests that PIN7-mediated auxin transport is manipulated in line with constitutive ABP1 overexpression.

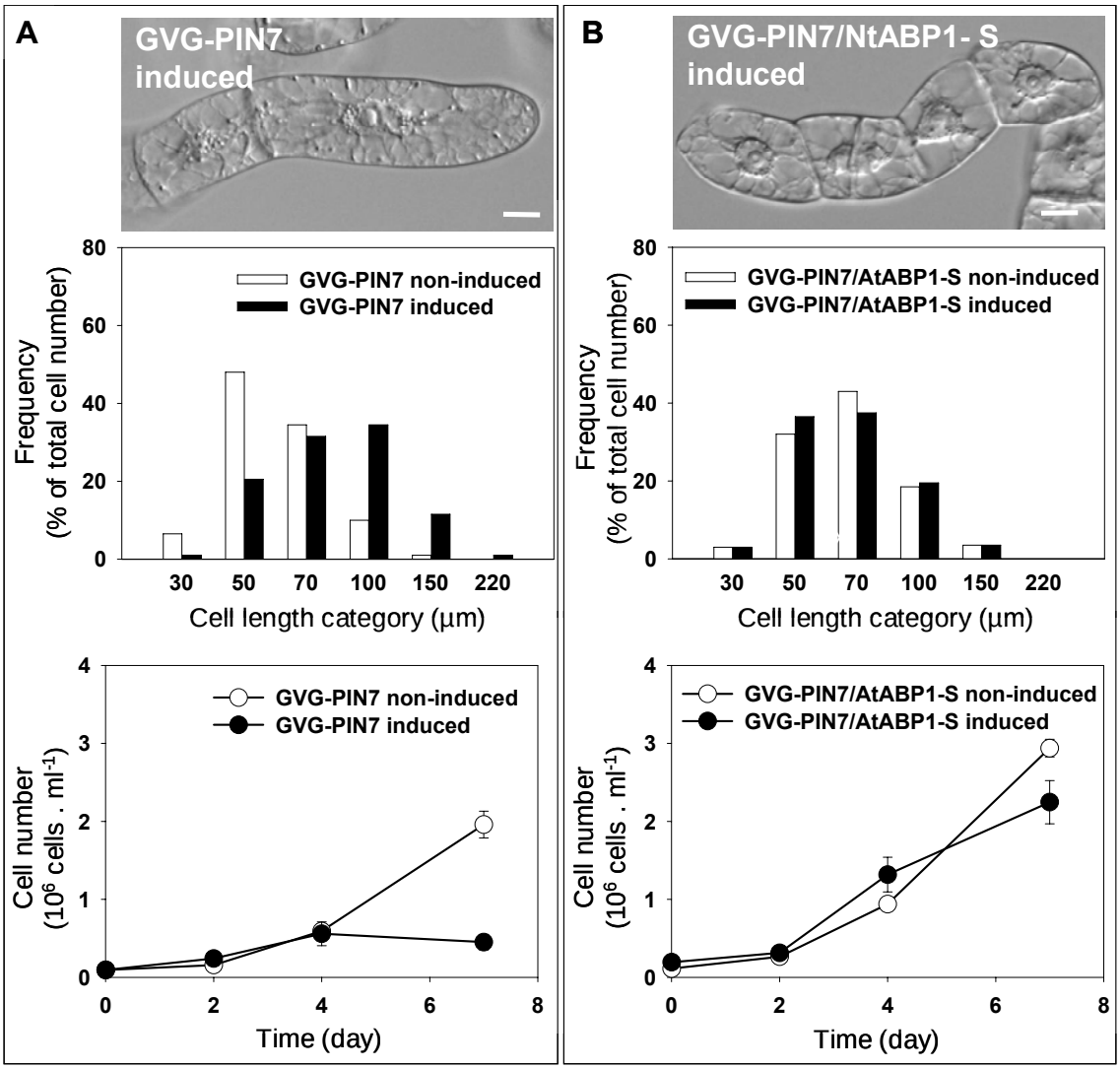

Figure 5.10: Cell growth parameters of GVG-PIN7 **(A)** and GVG-PIN7/NtABP1-S cells **(B)**. Nomarski DIC images, scale bars 20µm (upper panels), categorized cell length distribution (middle panels) and growth curves reflecting cell number (lower panels). The expression of GVG-PIN7 was induced by 1 µM dexamethasone. DMSO as solvent was added into control Error bars SEM (n=4).

To measure the effect of ABP1 on auxin efflux, the accumulation of radioactively labeled NAA reflecting activity of the active auxin efflux was measured in these lines. While the one-day dexamethasone-induced overexpression of *PIN7* alone in GVG-PIN7 line enhanced auxin efflux (i.e. decreased auxin accumulation), the *At*PIN7-dependent auxin efflux in induced GVG-PIN7/NtABP1-S line was strongly decreased (auxin accumulation was much higher) (Figure 5.11A). Moreover, as shown in Figure 5.11B, auxin efflux that was increased in induced GVG-PIN7 line, i.e. *At*PIN7-specific auxin efflux, was clearly NPA-sensitive. Although NPA also blocked auxin efflux in induced GVG-PIN7/NtABP1-S line, this cannot

be attributed to the PIN7 activity (as the level of auxin accumulation in the presence of ABP1 over-expressed was the same in both induced and non-induced lines) (Figure 5.11B).

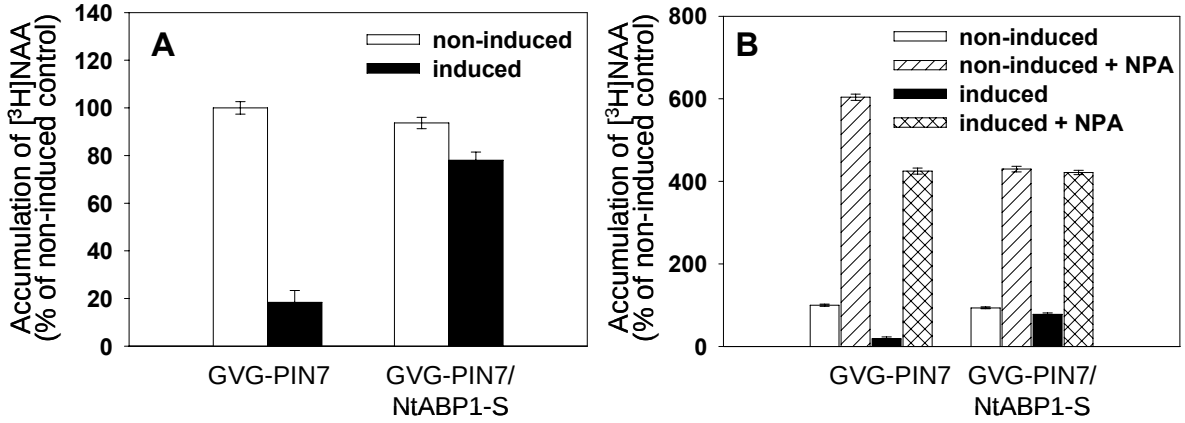

Figure 5.11: [^{3}H]NAA accumulation reflecting the activity of active auxin efflux. **(A)** [^{3}H]NAA accumulation in one-day-old GVG-PIN7 and GVG-PIN7/NtABP1-S, 20-min after addition of labeled auxin. **(B)** The effect of NPA on the [^{3}H]NAA accumulation. One-day-old GVG-PIN7 and GVG-PIN7/NtABP1-S treated with 10μM NPA at the time 20 min after addition of labeled NAA together with NPA. Data expressed as the percentage (corresponding untreated control=100%). Error bars show SEM (n=4). The expression of *PIN7* was induced by 1 μM dexamethasone and DMSO as solvent was added into control.

In agreement with the results of inducible *PIN7* overexpression on the background of constitutive ABP1 expression, opposite approach with inducible *AtABP1* on the background of constitutive *PIN1-GFP* showed the same results (Figure 5.12). Again, while constitutive expression of *PIN1-GFP* increased NPA-sensitive auxin efflux in comparison with control BY-2 cells (Figure 5.12A), there was less NPA-sensitive auxin efflux after the induction of *AtABP1* (Figure 5.12B).

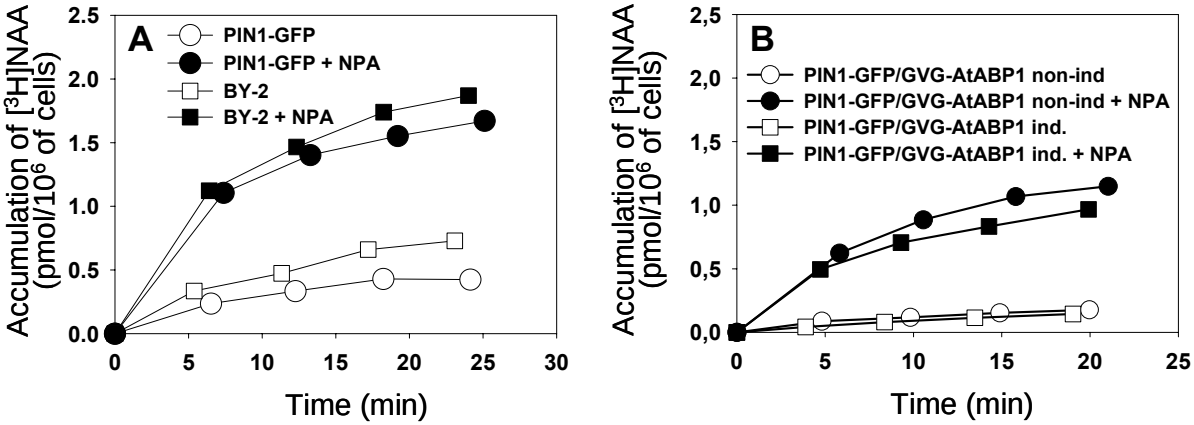

Figure 5.12: The effect of NPA on the [^{3}H]NAA accumulation in one-day-old BY-2, PIN1-GFP and PIN1-GFP/GVG-AtABP1 cells. **(A)** Kinetics of [^{3}H]NAA accumulation in one-day-old BY-2 and PIN1-GFP cells treated with 10μM NPA for the whole uptake period. **(B)** Kinetics of [^{3}H]NAA accumulation in one-day-old PIN1-GFP and PIN1-GFP/GVG-AtABP1 cells. The expression of *AtABP1* was induced by 1μM dexamethasone and DMSO as solvent

was added into control. 10μM NPA added at the beginning of the uptake period. Error bars show SEM (n=4).

Altogether, the auxin accumulation assays clearly showed that ABP1 is involved in modulation of the cell-to-cell auxin transport dependent on PIN proteins.

5.3 ABP1 affects vesicle trafficking processes related to dynamics of PIN proteins

To study the mechanism of ABP1-triggered changes in PIN-dependent auxin transport, PIN1-GFP/GVG-AtABP1 cells were used for *in vivo* confocal microscopy observation of PIN1-GFP dynamics after *ABP1* over-expression. The fluorescence recovery after photobleaching (FRAP) was studied in the rectangular regions of interests placed interactively at the plasma membrane (see Materials and Methods for details). As it is already suggested from the representative confocal images (Figure 5.13), the kinetics of FRAP indicated that after *ABP1* over-expression the recovery of PIN1 at the plasma membrane is slower (Figure 5.14A).

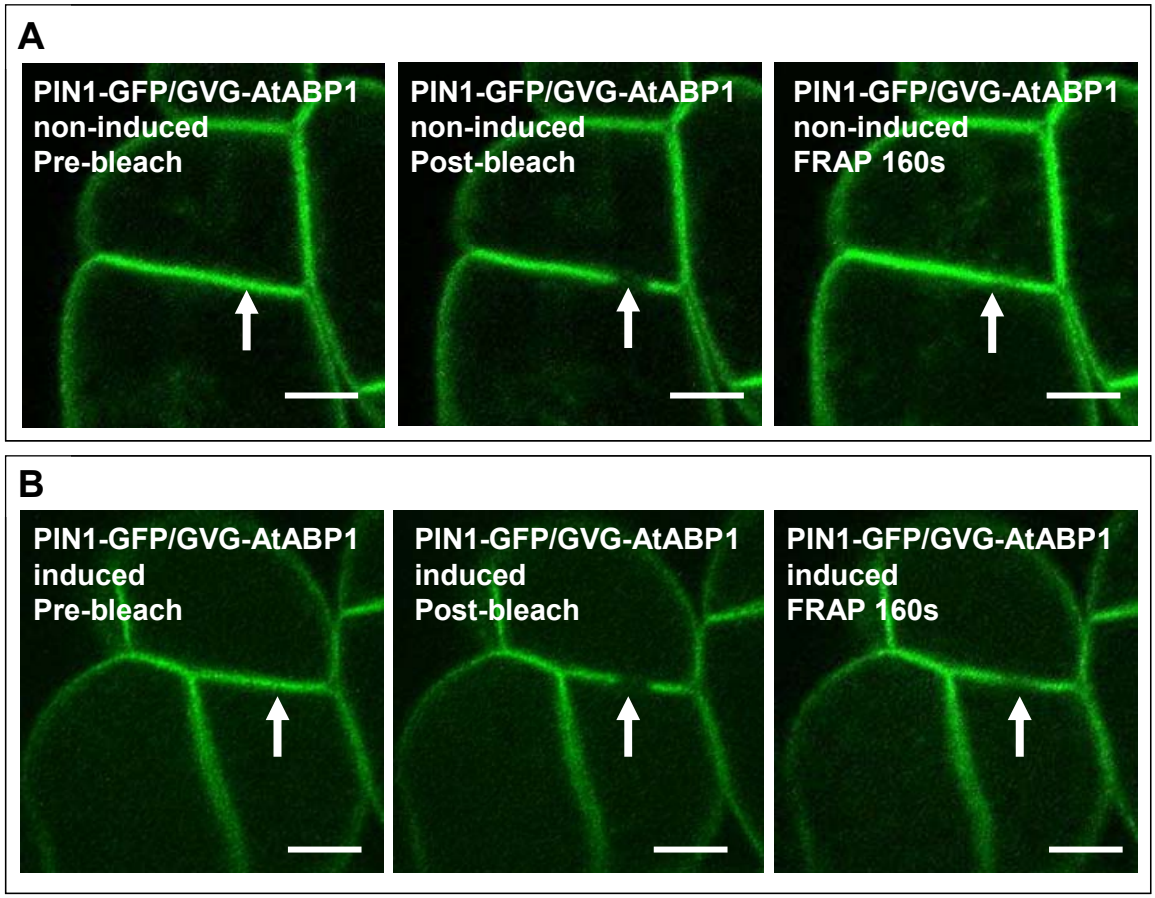

Figure 5.13: The fluorescence recovery after photobleaching (FRAP) in the plasma membrane of three-day-old PIN1-GFP/GVG-AtABP1 cells. **(A)** Non-induced cells. **(B)** Induced cells. The expression of *AtABP1* was induced by 1μM dexamethasone and DMSO as solvent was added into control. Scale bars 10 μm.

Inhibitors of membrane trafficking were used to dissect which step of PIN1-GFP intracellular dynamics is influenced by ABP1. After the treatment of cells with the inhibitor of anterograde protein trafficking brefeldin A (BFA) the effect of *ABP1* over-expression was the same as in untreated cells (Figure 5.14B) suggesting that ABP1 effect is independent of anterograde vesicle transport and that ABP1 rather stimulates endocytosis. This is further supported by the fact that tyrphostin A23, the inhibitor of recruitment of endocytic cargo (including PINs) into the clathrin-mediated pathway, prevented the effect of *ABP1* over-expression (Figure 5.14C). Slightly increased rate of FRAP after the *ABP1* over-expression was observed in cells treated with 5μM NAA (Figure 5.14D) supporting further the fact that endocytosis of PINs represents the target process for ABP1 action.

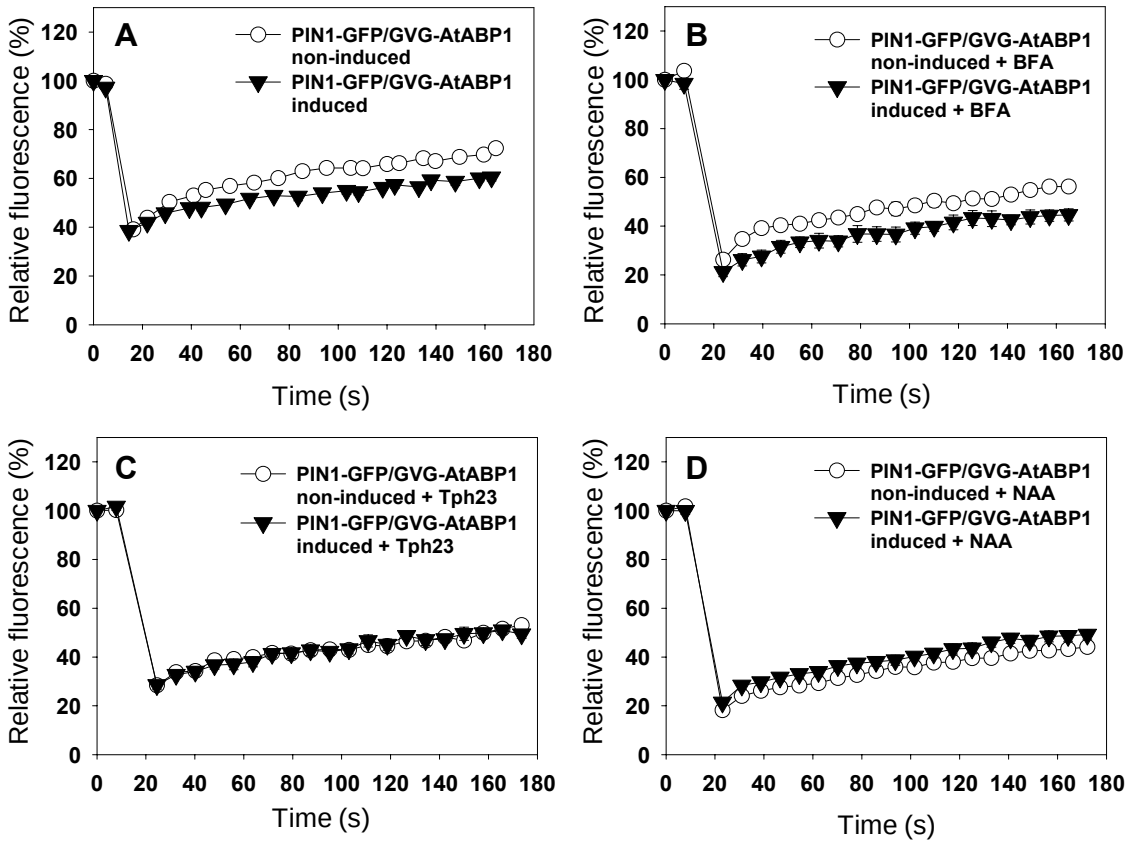

Figure 5.14: The kinetics of the fluorescence recovery after photobleaching (FRAP) in three-day-old PIN1-GFP/GVG-AtABP1 cells. **(A)** PIN1-GFP/GVG-AtABP1 non-induced and induced cells. **(B)** PIN1-GFP/GVG-AtABP1 non-induced and induced cells pretreated with 20μM BFA for 30 min. **(C)** PIN1-GFP/GVG-AtABP1 non-induced and induced cells pre-treated for 30 min with 50 μM Tyrphostin A23. **(D)** PIN1-GFP/GVG-AtABP1 non-induced and induced cells pre-treated with 5μM NAA for 1h. Expression of AtABP1 was induced by 1μM dexamethasone, DMSO as solvent was added into controls. Error bars SEM (n=8).

FRAP analysis of PIN trafficking has been supplemented with the auxin efflux assays addressing the effect of BFA. These assays showed that BFA increased auxin accumulation very effectively in cells, where ABP1 was not induced, i.e. in non-induced PIN1-GFP/GVG-AtABP1 and in GVG-PIN7 cells (Figure 5.15A, B, left columns). Upon induction of *AtABP1* in PIN1-GFP/GVG-AtABP1 (Figure 5.15A, right columns) as well as constitutive expression of *NtABP1-S* in GVG-PIN7/NtABP1-S cells (Figure 5.15B, right columns) the effect of BFA decreased.

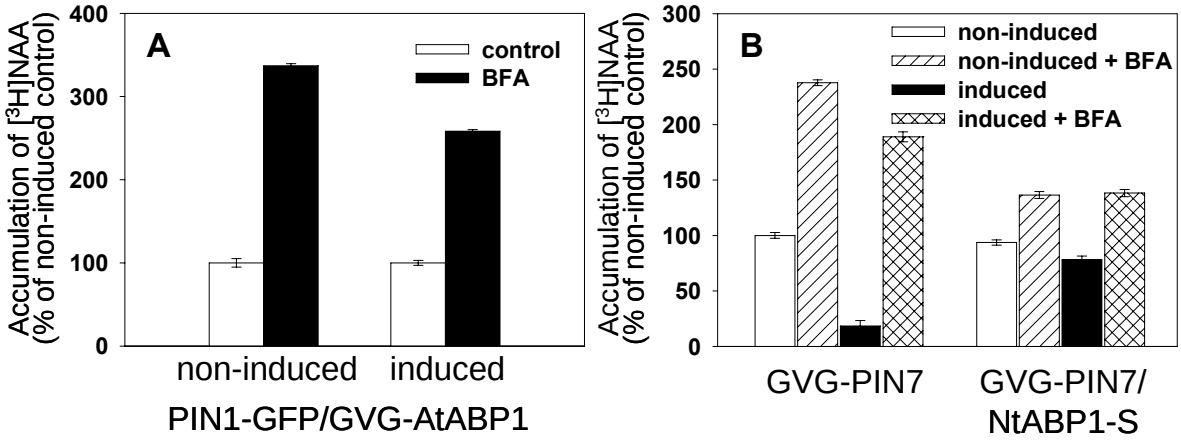

Figure 5.15: [^{3}H]NAA accumulation reflecting the effect of BFA on PIN-dependent auxin efflux in one-day-old ABP1-overexpressing cells. 20-min uptake period. **(A)** The effect of 20 µM BFA in PIN1-GFP/GVG-AtABP1 cells. **(B)** The effect of 20 µM BFA in GVG-PIN7 and GVG-PIN7/NtABP1-S cells. Error bars show SEM (n=4). The expression of *AtABP1* or *PIN7* was induced by 1µM dexamethasone and DMSO as solvent was added into control.

To analyze possible changes in signaling molecules at the plasma membrane mediating the signal induced by the activity of ABP1, products of activities of plasma-membrane-associated phospholipases has been analyzed in GVG-PIN7/NtABP1-S cells. After loading cells with bodipy-PC for two hours, levels of typical products lysophosphatidyl choline (LPC), phosphatidic acid (PA) and diacylglycerol (DAG) were determined. The level of LPC, indicating indirectly the activity of PLA2, decreased after induction of ABP1 in both PIN7 induced and non-induced cells (Figure 5.16A). The removal of 2,4-D had no effect. The level of PA, indicating indirectly the activity of PLD, was slightly increased (Figure 5.16B) and removal of 2,4-D surprisingly increased its level massively only after overexpression of *ABP1* alone, but not together with *PIN7*. The level of DAG, indicating indirectly the activity PC-PLC, was increased after induction of ABP1 in both PIN7 induced and non-induced cells (Figure 5.16C). Here the auxin wash-out reduced this increase only in induced cells.

Altogether, data form FRAP, auxin efflux assays with vesicle trafficking inhibitors and determinations of plasma-membrane-associated phospholipase activities suggest that ABP1 controls intercellular auxin transport by triggering trafficking of vesicles with PIN auxin efflux carriers. At non-genomic level, the activities of phospholipases are influenced by the activity of ABP1.

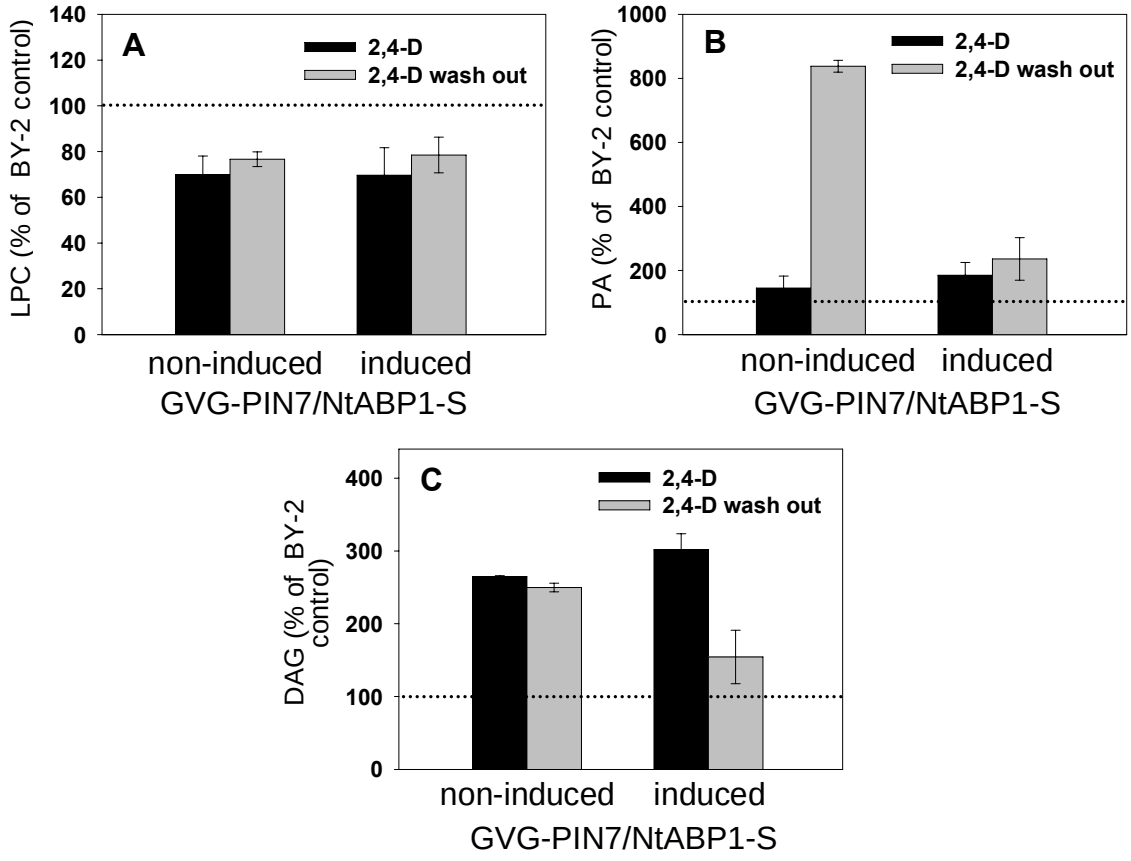

Figure 5.16: Relative levels of products of plasma-membrane-associated phospholipases. Three-day-old GVG-PIN7/NtABP1-S cells cultured in standard medium with 1μM 2,4-D. Wash-out of auxin immediately before the addition of BODIPY-PC for two hours, fluorescent products separated using TLC. The expression of *PIN7* was induced by 1 μM dexamethasone and DMSO as solvent was added into control. Error bars show SEM (n=2). **(A)** Relative levels of LPC. **(B)** Relative levels of PA. **(C)** Relative levels of DAG.

5.4 ABP1 mediates processes of intracellular auxin sequestration and metabolism

Following the same idea as presented in chapter 5.2, where the role of ABP1 in plasmamembrane-associated PIN7- and PIN1-dependent auxin transport was dissected, ABP1 was studied also with respect to the activity of the intracellular ER-localized PIN5 auxin transporter. It has been reported (Mravec et al., 2009) that the activity of PIN5 increases the conjugation of IAA in the ER and decreases the intracellular pool of free IAA. So, tobacco BY-2 cells were double-transformed with tobacco *ABP1* under 35S constitutive promoter and

dexamethasone-inducible Arabidopsis *PIN5* (GVG-PIN5). Single tobacco line GVG-PIN5 was described earlier (see Materials and Methods). Resulting double-transformed line was verified using RT-PCR (Figure 5.17) and designated GVG-PIN5/NtABP1-S. The objective was to address the role of ABP1 in cells, where intracellular auxin transport through PIN5 protein is enhanced.

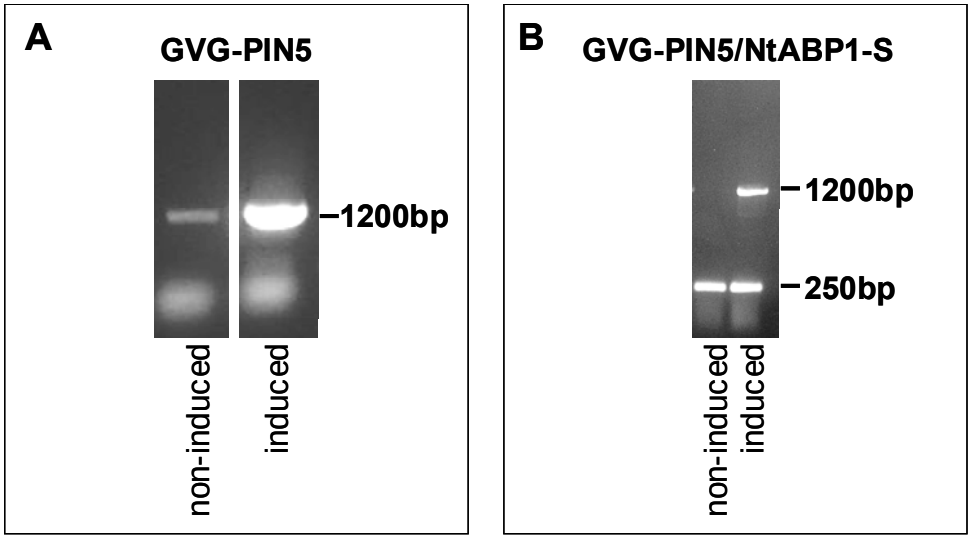

Figure 5.17: RT-PCR verification of transgene expression in GVG-PIN5 and GVG-PIN5/NtABP1-S cells induced with 1 μM dexamethasone for 24 hours. **(A)** Inducible PIN5 expression in GVG-PIN5 cells (1200 bp fragment). **(B)** Inducible *PIN5* expression (1200 bp fragment) at the background of constitutive NtABP1-S (250bp) in GVG-PIN5/NtABP1-S cell line.

As reported earlier (Skůpa et al., 2008), GVG-PIN5 cells upon induction with dexamethasone were gradually dying (Figure 5.18C) and cell division activity was strongly decreased (Figure 5.18F). This phenomenon was observed also in GVG-PIN5/NtABP1-S cells, but in addition to this, individual cell isodiametric expansion was increased (Figure 5.18D). There was about 20% of oversized cells with diameter more than 70μm (Figure 5.19B). Interestingly, in comparison with PIN7-dependent cell elongation (Figure 5.10A), the ABP1-promoted expansion was only isodiametric (Figure 5.19B). Again in contrast to PIN7-promoted elongation, this expansion effect was not reversible with NPA (Figure 5.18E and 5.19C) suggesting that ABP1-induced cell expansion on the background of PIN5 activity is triggered from the cell interior. Overall cell division rate was impaired to the same extent in both GVG-PIN5 and GVG-PIN5/NtABP1-S cells (Figure 5.18F).

These results suggest that ABP1 somehow distinguishes not only lower amount of auxin inside the cell, but importantly the direction of its flow.

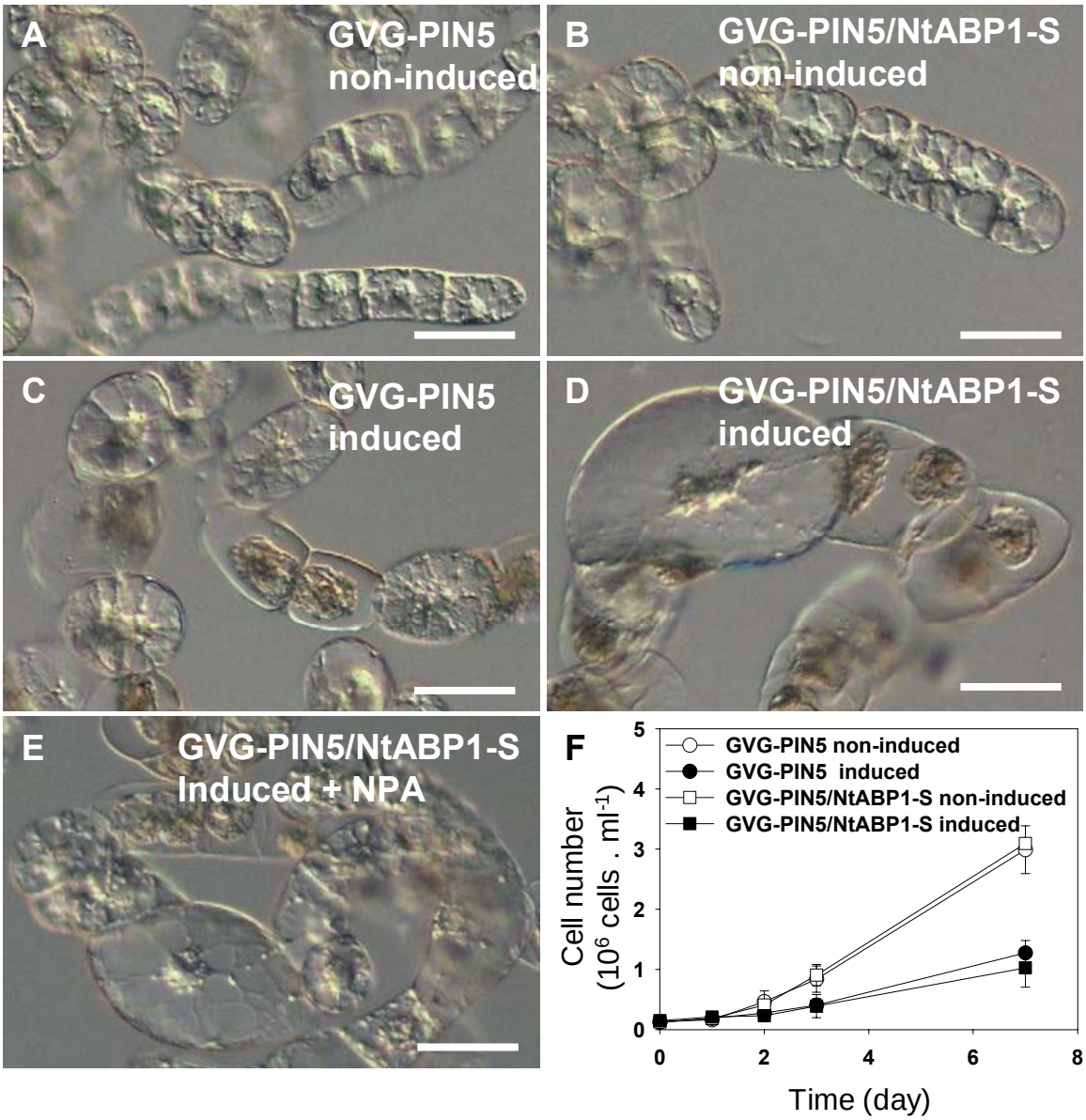

Figure 5.18: Cell growth parameters of GVG-PIN5 **(A, C)** and GVG-PIN5/NtABP1-S **(B, D)** cells. **(E)** The effect of 10 µM NPA in GVG-PIN5/NtABP1-S cells. Nomarski DIC images of 3-day-old cells, scale bars 50µm. **(F)** Growth curve reflecting cell number. The expression of *PIN5* was induced by 1 µM dexamethasone. DMSO as solvent was added into control Error bars SEM (n=4).

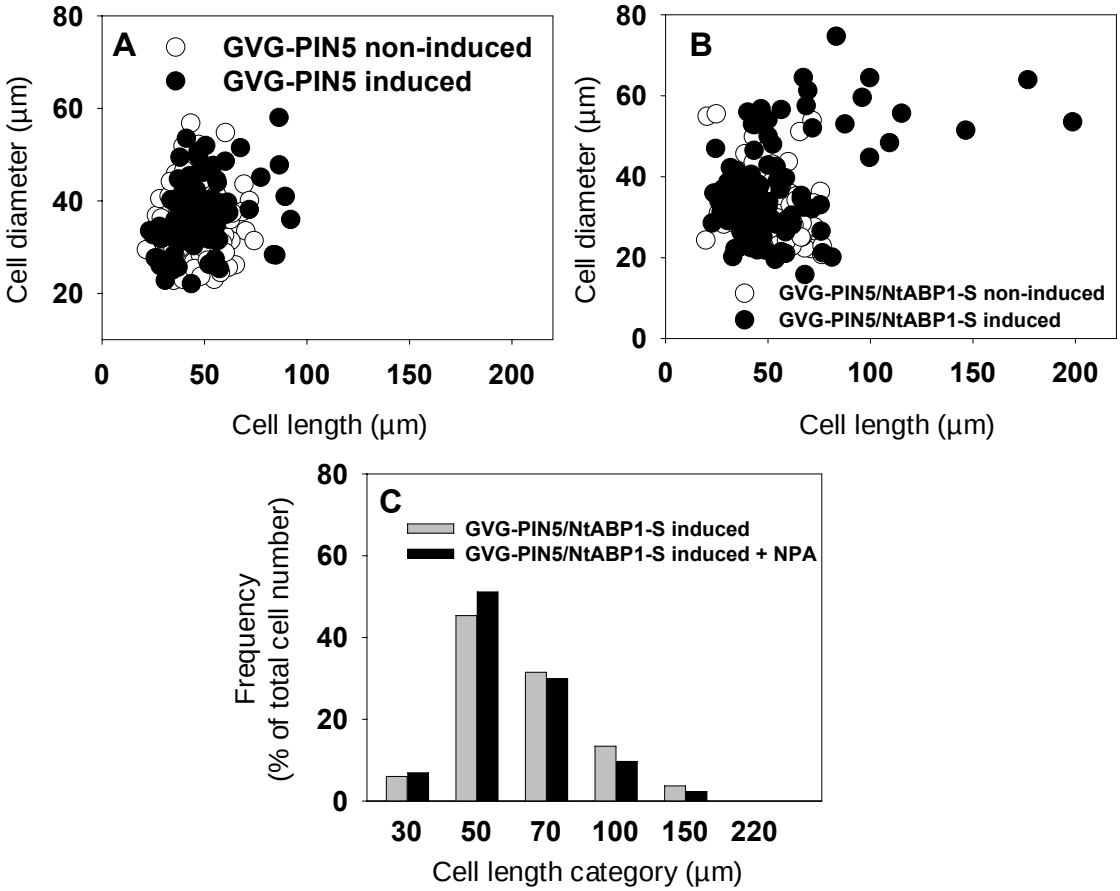

Figure 5.19: Reciprocal plot of cell dimensions **(A, B)** and categorized cell length distribution after 10 µM NPA treatment **(C)** of three-day-old GVG-PIN5 and GVG-PIN5/NtABP1-S cells. The expression of *PIN5* was induced by 1 µM dexamethasone. DMSO as solvent was added into control.

The analysis of cell viability showed that in standard 2,4-D-containing medium around one quarter of all cells was dying upon *PIN5* overexpression and the presence of ABP1 did not influence this parameter (Figure 5.20A). However, ABP1 activity seems to protect cells from cell death when the cells were cultured in the presence of 1µM IAA, where cell death in control cells is even promoted (Figure 5.20B). This suggests the activity of ABP1 in retention of free (i.e. non-conjugated) form of IAA being available for sustainable cell division and growth.

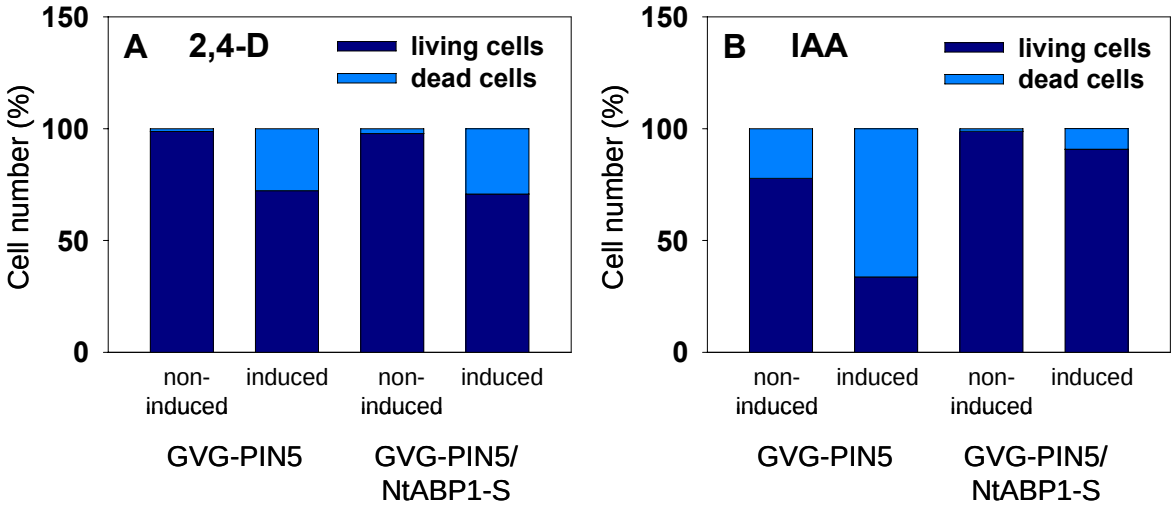

Figure 5.20 Viability of three-day-old GVG-PIN5 and GVG-PIN5/NtABP1-S cells cultured in standard medium containing 1μM 2,4-D **(A)** or 1 μM IAA **(B)**. The expression of *PIN5* was induced by 1μM dexamethasone. DMSO as solvent was added into control.

To address the role of ABP1 in the intracellular IAA management, IAA metabolic profiles were compared in BY-2, NtABP1-S, GVG-PIN5 and GVG-PIN5/NtABP1-S cells. As shown in Figure 5.21, IAA metabolic profiles of BY-2 cells and cells constitutively over-expressing tobacco *ABP1* gene for one day (NtABP1-S cells) were almost the same (Figure 5.21A). The overproduction of PIN5 for one day resulted in almost total conversion of free IAA into conjugates and a predominant, yet unidentified metabolite was formed with retention time 8.7 min (Mravec et al., 2009 and Figure 5.21B). However, the over-expression of ABP1 was able to prevent a significant portion of free IAA from its conjugation even in the presence of the over-expressed PIN5 (Figure 5.21C). This suggests that the action of ABP1 (either on the plasma membrane or intracellular ER-localized) helps to keep the pool of free IAA necessary for cell division and growth.

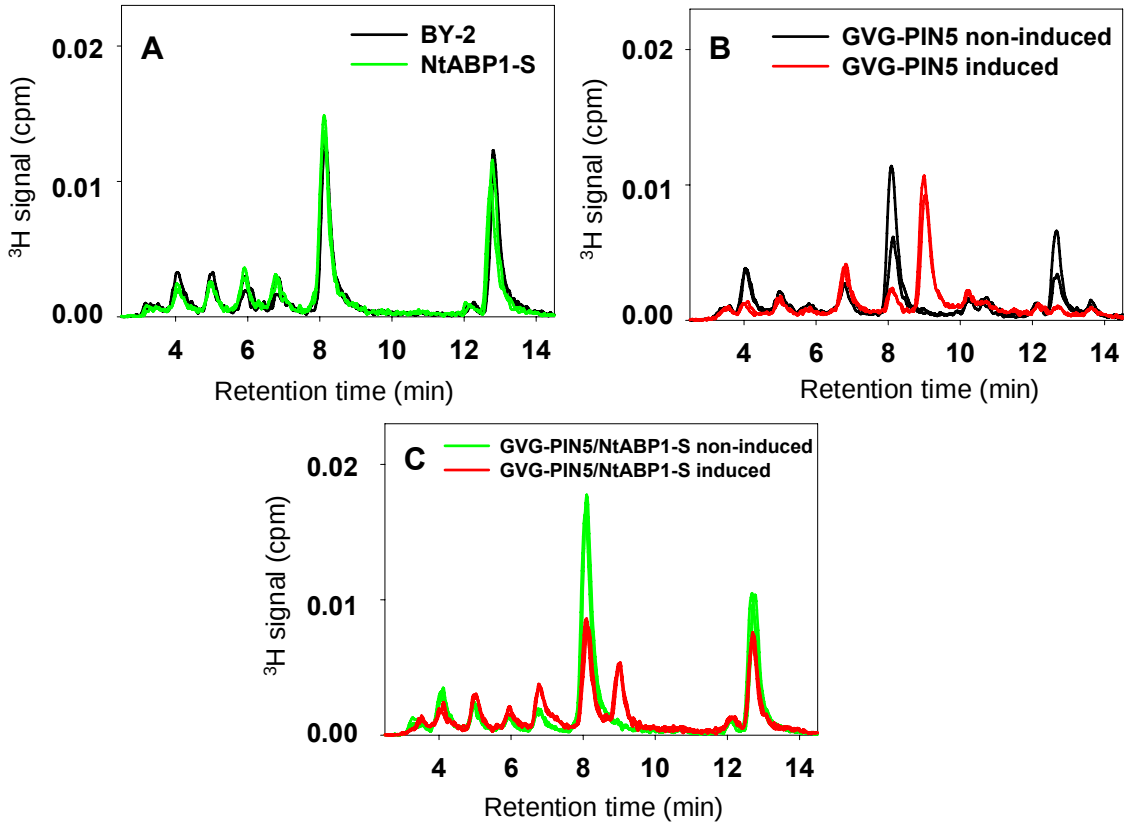

Figure 5.21: The HPLC chromatogram of IAA metabolic profile in one-day-old cells treated with ^{3}H-IAA for 2.5h. Retention time of the peak of free IAA and the IAA-glucose conjugate is 12.5 min and 8 min, respectively. *PIN5* expression was induced with 1 μM dexamethasone and DMSO as solvent was added into control. **(A)** BY-2 and NtABP1 cells. **(B)** The effect of *PIN5* overexpression in GVG-PIN5 cells (see also Mravec et al. 2009). **(C)** The effect of *PIN5* overexpression in GVG-PIN5/NtABP1-S cells.

6 Discussion

Auxin binding protein 1 mediates both cell division and cell expansion in tobacco BY-2 cells

In the first part of this thesis the processes of cell division and cell expansion are addressed with respect to the ABP1 function. Growth characteristics of BY-2 suspension cells (cell length categories, number of cells in cell chains) as well as phenotype and division rate in lines over-expressing tobacco ABP1 in sense or antisense orientations or conditionally over-expressing Arabidopsis ABP1 were determined.

The over-expressed ABP1 itself did not alter cell size, growth rate or the distribution of cell files categories of cells in the exponential growth phase grown under standard conditions of cultivation. Standard cultivation medium for BY-2 cells contains synthetic auxin 2,4-D, which is needed for cell division activity; another synthetic auxin NAA can support cell elongation (Hasezawa and Syono, 1983; Nagata et al., 1992). Naturally occurring IAA and synthetic NAA are better substrates than 2,4-D for ABP1-mediated physiological effects such as cell expansion (Steffens & Lüthen, 2000). However, increased cell expansion was reported in tobacco plants over-expressing *Arabidopsis* ABP1 and in maize cell cultures over-expressing maize ABP1 in response to high levels of 2,4-D (Jones et al., 1998) suggesting certain activity of 2,4-D as well. In agreement with this, the results presented here showed weak ABP1 over-expression-dependent increase of the cell size only at higher concentrations of 2,4-D in cultivation medium. Chen et al. (2006) reported on the altered sensitivity to NAA of tobacco calli over-expressing maize ABP1. As already shown by Hasezawa nad Syono (1983) by scoring cell elongation and division frequency during the regeneration of BY-2 cells from protoplasts, lower amount of NAA is sufficient for cell elongation, while higher amount of NAA stimulated cell division. BY-2 cells show this dual auxin pathway, but as noticed by Jones et al. (2004), 2,4-D is the exception in the perfect match between the binding affinity of ABP1 to various auxins and their ability to stimulate cell division. NAA and 2,4-D might even stimulate different pathways for the regulation of cell elongation and cell division as shown in another tobacco cell line VBI-0 (Campanoni and Nick, 2005) with ABP1 involved exclusively in NAA-induced cell elongation. Jones and Herman (1993) showed higher amounts of ABP1 in culture medium when maize BMS cells were cultivated without auxin for 72 h. According to this it could be speculated that cells over-expressing *ABP1* could be more tolerant to lower levels of 2,4-D in the cultivation medium. However, this was not confirmed in the experiments presented here as the cells over-expressing *ABP1* did not differ

from control line after lowering exogenous auxin. Nevertheless, it has been shown here that the function of ABP1 in BY-2 cells is indeed essential. The antisense ABP1 inhibition of cell growth observed in our experiments supports the results of Chen et al. (2001a and b), where no cell expansion and aberrant cell divisions, respectively, were observed, and also the results of David et al. (2007), where delayed G2/M transition was observed.

In our experiments tobacco ABP1 was immunolocalized in IAA-treated elongating cells at the plasma membrane, while in 2,4-D-treated dividing cells it was localized exclusively to the ER. This unexpected fact uncovers very promising field for immunofluorescence tracking of ABP1 distribution in various plant tissues with differential elongation status. It also points to the fact that ABP1 localization at the plasma membrane is required for cell elongation. Although there is also a possible influence of auxin on the ABP1 plasma membrane deposition, Henderson et al. (1997) did not observe enhanced plasma membrane localization of ABP1 in maize roots treated with auxin. These authors applied 10 μM 2,4-D and observed increase in Golgi apparatus volume induced by auxin treatment but not higher plasma membrane localization of ABP1. However, as already mentioned above, 2,4-D is not a good substrate for ABP1 (Löbler and Klambt, 1985a, b), and therefore ABP1 plasma membrane localization may not be inevitable under these conditions. On the other hand, the clustering of ABP1 at the surface of maize protoplasts after application of 10 μM IAA or 10 μM NAA was detected by silver-enhanced immunogold procedure and it was shown not to be evoked by inactive auxin analogs (Diekmann et al., 1995). Also in tobacco evacuolated protoplasts the electrical response to NAA combined with polyclonal antibodies directed against ABP1, that are unlikely to enter the cell, showed that this response resides only at plasmalemma (Barbier-Brygoo et al., 1989). Likewise, Steffens et al. (2001) proved that the auxin signal resulting in swelling of protoplasts from corn coleoptiles and *Arabidospsis* hypocotyls is perceived by extracellular ABP1.

All these results point to the differential roles of various auxins, namely 2,4-D on one side and IAA and NAA on the other one, in cell development as well as to the importance of subcellular localization of ABP1 in relation to particular processes of cell development.

ABP1 mediates intercellular auxin transport

The appearance of aberrant cell divisions in Arabidopsis homozygous mutant embryos (Chen et al., 2001b) suggests that this phenotype could be related to impaired polar auxin transport. The formation of cell clusters observed in our experiments after the expression of anti-sense ABP1 also pointed indirectly to the altered intercellular polar auxin transport as

BY-2 cells are dependent on exogenous auxin application. Disturbed polarity of cell division in cell files after the application of inhibitors of auxin efflux were reported by Petrášek et al. (2002) and Campanoni et al. (2003) in VBI-0 tobacco cells and Dhonukshe et al. (2005) in BY-2 cells. Therefore, it seems that auxin level in BY-2 cells and the polarity of cell division depends largely on auxin transport from cell to cell. With respect to the role of ABP1, our cell phenotypic analysis as well as auxin accumulation assays clearly showed that cell overexpressing ABP1 were less sensitive to NPA, thus connecting ABP1 action and polar auxin transport machinery. Interestingly, this thesis brings evidence on the differential action of two auxin efflux inhibitors NPA and PBA with respect to the role of ABP1. In contrast to NPA, the application of PBA had no detectable effect on cell phenotype related to ABP1 overexpression. PBA has been reported to affect auxin transport by influencing the actin dynamics (Dhonukshe et al., 2008). The effect of NPA on actin seems to be of secondary character, because NPA primarily and at much lower concentration affects the activity of auxin efflux carriers at the plasma membrane. Increasing amount of auxin inside the cell promotes actin bundling and consequently decreases deposition of new auxin carriers to the membrane; this in turn increases the intracellular amount of auxin. This regulatory circuit has been suggested for the auxin-actin regulation of cell-to-cell transport of auxin (Nick et al., 2009). This notion in relation to the results presented here means that ABP1 doesn't influence actin but more likely changes plasma membrane conditions leading to the altered deposition of auxin efflux transporters accessible to NPA.

Although the ability to accumulate auxin of the cell line conditionally overexpressing *Arabidopsis ABP1* is not significantly altered in comparison with non-induced control, ABP1 has been shown to affect auxin transport in cells, where PIN7-mediated auxin efflux was strongly upregulated. Most members of PIN protein family facilitate cell-to-cell auxin flow and they are the main determinants of polar transport of IAA in plant tissues (Petrášek et al., 2006; Petrášek and Friml, 2009). Plasma-membrane-residing PINs catalyze auxin efflux, as shown in BY-2 cells where the overexpression of *At*PIN7 evokes excessive auxin efflux (Petrášek et al., 2006). This excessive auxin efflux induces the auxin starvation phenotype (Winicur et al., 1998; Sakai et al., 2004), characterized by the inhibition of cell division, stimulation of cell elongation and amyloplast formation (Petrášek and Zažímalová 2006; Mravec et al., 2008). Importantly, cells over-expressing *ABP1* together with PIN7 showed normal phenotype and all auxin starvation symptoms were rescued in this line. This rescue was observed in our experiments after both inducible and constitutive ABP1 over-expression as well as after inducible PIN over-expression together with ABP1 constitutive expression.

This is important, because the origin of the lines is independent and the strategy opposite. The rescue effect is similar to the rescue of exaggerated PIN-dependent auxin efflux seen after NPA application (Mravec et al., 2008).

The auxin accumulation assays showed that in cells over-expressing *ABP1* together with PIN7 or PIN1 the accumulation of [^{3}H]NAA is not so increased after NPA application as in controls. This decreased sensitivity to NPA could point to the fact that ABP1 somehow influences the amount of auxin efflux carriers at the plasma membrane. Less auxin efflux carriers on plasma membrane could mean fewer target sites for NPA action. This was confirmed again by both approaches, i.e. with induced PIN on the background of stable over-expression of ABP1 and with induced ABP1 on the background of over-expression of PIN1. On the other hand, as shown by Tromas et al. (2009), NPA was able to disturb the auxin maxima in *Arabidopsis* roots independently of ABP1 activity. Therefore, more experiments are needed to explain this discrepancy.

ABP1 affects vesicle trafficking processes related to dynamics of PIN proteins

Since *ABP1* over-expressing cells are less sensitive to NPA and the overexpression of ABP1 decreases exaggerated PIN-dependent auxin efflux, ABP1 may act on PIN protein intracellular dynamics. PINs are known to undergo dynamic recycling between plasma membrane and endosomal compartments (Kleine-Vehn et al., 2008). The retrograde trafficking of PINs is clathrin-dependent (Dhonukshe et al., 2007) and depends also on sterol composition of the plasma membrane (Men et al., 2008; Willemsen et al., 2003). The polar delivery of PINs to plasma membrane is regulated by guanine nucleotide exchange factor for ADP-ribosylation factors (ARF-GEF) (Kleine-Vehn et al., 2008) and their localization to apical or basal end of cells also depends on their phosphorylation status (Michniewicz et al., 2007). Auxin itself is known to inhibit PIN endocytosis thus promoting its own transport (Paciorek et al., 2005). Athough the mechanism of this probably non-genomic auxin effect is not fully understood, it seems that ABP1 might be involved in this process (Robert et al., 2010). Thus, the regulation of PIN dynamics and vesicle trafficking may belong to these non-genomic rapid auxin responses that are assisted by ABP1.

To uncover the mechanisms by which ABP1 affects the auxin transport across membrane, *in vivo* confocal microscopy of PIN1-GFP was performed. BY-2 cell line over-expressing constitutively plasma membrane auxin carrier from the *Arabidopsis* PIN family in GFP fusion (PIN1-GFP) together with the inducible Arabidopsis ABP1 was used. Indeed, the over-expressed *ABP1* affected PIN1-GFP dynamics as shown by FRAP measurements. The

dynamics of FRAP of PIN1-GFP is in agreement with published data (Dhonukshe et al., 2007; Kleine-Vehn et al., 2008). The experiments were focused on the first three minutes of fluorescence recovery. Since the over-expression of ABP1 decreased the speed of FRAP, there are in principle three scenarios to explain this effect: ABP1 may influence the endocytosis of membrane vesicles, or their deposition to the plasma membrane, or both these processes in parallel. To test these possibilities, inhibitors of anterograde (BFA) and retrograde (tyrphostin A23) vesicle transport were used. After the treatment of cells with BFA the effect of *ABP1* over-expression was the same as in untreated cells suggesting that ABP1 effect is independent of anterograde vesicle transport and that ABP1 is more likely to stimulate endocytosis. This is further supported by the fact that the inhibitor of the recruitment of endocytic cargo into the clathrin-mediated pathway (including PINs), tyrphostin A23, prevented the effect of ABP1 over-expression. Slightly increased rate of FRAP after the ABP1 over-expression was observed in cells treated with NAA supporting further the idea that endocytosis of PINs represents the target process for the ABP1 action. The fact, that at higher auxin concentrations ABP1 seems to mediate the inhibition of PIN endocytosis suggests that ABP1 may have dual function depending on different auxin levels. This is in agreement with the results published by Robert et al. (2010). According to the results presented here and results of Robert et al. (2010) ABP1 may either promote endocytosis of PINs under low levels of auxin to retain auxin inside cells or to inhibit endocytosis of PINs under high levels of auxin to stimulate the export of auxin from cells.

Interestingly, the BFA treatment in cell lines over-expressing *ABP1* leads to lower auxin accumulation than would be expected. If ABP1 promotes endocytosis and BFA blocks exocytosis, it should result in even higher intracellular auxin accumulation, due to blocked delivery of new efflux carriers to the plasma membrane and ABP1-promoted endocytosis of the original ones from the plasma membrane. The explanation could be that higher concentration of auxin in the cell following BFA treatment provides the signal for ABP1 not to promote PIN endocytosis. In fact, this is consistent with the situation after NPA application when cells over-expressing ABP1 are less sensitive to NPA. The reason can be the same: after application of the inhibitor of auxin efflux the auxin concentration in the cell is enhanced thus providing the signal for ABP1. Since the physiological role of ABP1 during cell expansion was proved on plasma membrane (Rück et al., 1993, Leblanc et al., 1999) it is expected that upon binding of auxin, ABP1-triggered signal for PIN recruitment to the endocytotic vesicles is likely to take place at the plasma membrane as well. Therefore, the involvement of activity of plasma-membrane-associated phospholipases could be expected. Three types of

phospholipases were shown to be related to auxin signaling (see Introduction). Fluorescently labeled phosphatidyl choline as a substrate for the phospholipases was added to BY-2 cells over-expressing tobacco ABP1 alone or together with PIN7, both in the presence or absence of auxin. This approach could help answering the question whether the auxin signal is passed from ABP1 via the phospholipases to control vesicle trafficking of PIN proteins. The analysis of products of activity of the plasma-membrane-associated phospholipases suggested that their action, previously reported to be important for the trafficking of PINs, is influenced after *ABP1* over-expression. In spite of the fact that the experiments showed interesting ABP1-specific PA production after the removal of auxin, at this moment they are preliminary to make conclusions related to this ABP1 action.

ABP1 mediates processes of intracellular auxin sequestration and metabolism

Experiments using the line over-expressing *At*PIN5 pointed to the unexpected role of ABP1 in the intracellular auxin management . PIN5 belongs among three PINs from *Arabidopsis* PIN protein family that have much shorter cytosolic loop (Mravec et al., 2009). It seems to reside at the membrane of ER and it has been suggested to facilitate auxin translocation from cytosol into the lumen of ER. Since the ER contains enzymic apparatus for the auxin conjugation it has been suggested that PIN5 contributes to the regulation of auxin homeostasis inside cells (Mravec et al., 2009). Over-expression of *PIN5* in BY-2 cells resulted in the arrest of cell division, slight cell expansion and programmed cell death (Skůpa et al., 2008). When *ABP1* was over-expressed together with *PIN5* the cells were still dying but they also started to expand. The fact that NPA application was not rescuing this action suggests that it is not due to cell-to-cell auxin transport. The cell expansion and amyloplast formation are the hallmarks of auxin depletion/starvation. In this case, it can be caused by higher transport of auxin to the ER. Low level of auxin in cytoplasm could then be a signal for the ABP1-mediated cell expansion, triggered possibly from outside the cell. In contrast to PIN7-dependent intercellular polar auxin transport, the overexpression of *PIN5* influences auxin distribution inside the cell, thus the expansion occurs isodiametrically. These results suggest that ABP1 may be able to distinguish not only lower amount of auxin inside the cell, but also the direction of its flow. Whereas under standard conditions the over-expression of *ABP1* does not influence the cell death stimulated by the PIN5 over-expression, cultivation of these cells in IAA instead of 2,4-D partly rescues their viability.. Importantly, the viability of GVG-PIN5 cells is lower when cultured in the medium supplied with IAA . Since IAA is metabolized faster then 2,4-D in tobacco cells (Delbarre et al.1996), the cells of GVG-PIN5

line cultivated in IAA were dying faster. Our IAA metabolic profiling suggested that ABP1 is essential also for the maintenance of auxin homeostasis by preventing the auxin conjugation and keeping certain pool of free IAA available, as over-expressed *ABP1* in GVG-PIN5/NtABP1-S line maintains some IAA in non-metabolized form. In agreement with this, Chen et al. (2006) observed higher auxin metabolism and its conjugation in particular, in ABP1-antisense BY-2 cells.

ABP1 enigma continues

All these data indicate that the role of ABP1 is essential for the control of cell expansion, which is part of the growth cycle of tobacco BY-2 cell line. ABP1 is also regulating the PIN-dependent cell-to-cell auxin transport, probably via PIN endocytosis. It also contributes to sustain intracellular auxin homeostasis by preventing IAA conjugation in the ER.

However, important questions remain unanswered. Does the subcellular localization of ABP1 determine its particular action? Or is it either the ER- or the plasma-membrane-localized ABP1, which mediates all these processes? Is it the auxin concentration inside the cell which triggers ABP1-mediated actions? Anyway, there are no doubts that ABP1 is an important player in auxin management in plant cells.

7 Conclusions / Závěry

7.1 Conclusions

The role of AUXIN BINDING PROTEIN 1 (ABP1) in the auxin management in plant cells was followed using simplified model material of suspension-cultured cells of tobacco BY-2 line. ABP1 is a putative auxin receptor considered to mediate fast non-genomic responses to auxin and it can be involved in every aspect of the regulation of auxin responses, metabolism and transport. There are four major conclusions that could be made based on the results presented in this thesis:

1) Auxin binding protein 1 mediates both cell division and expansion in tobacco BY-2 cells. In standard cultivation conditions or at lower concentrations of 2,4-D in culture medium, ABP1 overexpression had no detectable impact on cell division, cell elongation or cell growth.. 5-times increased 2,4-D concentration stimulated weakly cell elongation. . Antisense suppression of *ABP1* expression resulted in disturbance in both cell expansion and cell division intensity, suggesting that ABP1 is essential for the control of balance between cell division and cell elongation during the growth cycle. ABP1 is localized in endoplasmic reticulum of cells cultivated in standard medium supplemented with 1 μM 2,4-D and it appeared also at the plasma membrane following the IAA application.

2) ABP1 mediates intercellular auxin transport. Cells over-expressing ABP1 were less sensitive to NPA, the inhibitor of auxin efflux carrier activity on plasma membrane, but not to PBA, the inhibitor of actin dynamics. ABP1 over-expression rescued the auxin starvation phenotype induced by the over-expression of the plasma-membrane-localized PIN auxin efflux carriers and decreased the *At*PIN-mediated auxin efflux. Altogether, these findings suggest that ABP1 affects the active auxin efflux.

3) ABP1 affects vesicle trafficking processes related to PIN proteins dynamics. FRAP measurements revealed that *ABP1* inducible over-expression slowed down the PIN1-GFP fluorescence recovery at the plasma membrane. Selective application of inhibitors of anterograde vesicle trafficking (BFA) and endocytosis (Tyrphostin A23) showed that that ABP1 action was independent of anterograde vesicle transport and it stimulated endocytosis of PIN1-GFP. The *ABP1* over-expression also influenced activity of the plasma-membrane-associated phospholipases, previously reported to be important for the trafficking of PINs.

4) ABP1 mediates processes of intracellular auxin sequestration and metabolism. In the presence of IAA in cultivation medium, ABP1 rescued the effects caused by the over-expression of the endoplasmic-reticulum-localized transporter *At*PIN5 and protected free IAA

from conjugation. Thus, ABP1 contributes to the maintenance of intracellular auxin homeostasis by preventing the PIN5-dependent metabolic changes of IAA.

7.2 Závěry

Úloha AUXIN BINDING PROTEIN 1 (ABP1) v hospodaření rostlinných buněk s auxinem byla studována s využitím zjednodušeného modelového materiálu suspenzní kultury tabáku BY-2. ABP1 je předpokládaným receptorem pro auxin, který zprostředkovává rychlé odpovědi k auxinu nezávislé na expresi genů, a může být součástí veškerých procesů souvisejících s regulací odpovědí na auxin, jeho metabolismu a transportu. Z výsledků uvedených v této práci plynou čtyři hlavní závěry:

1) Auxin binding protein 1 je nezbytný pro dělení i růst buněk suspenzní kultury BY-2. Za běžných kultivačních podmínek ani za podmínek sníženého obsahu 2,4-D v kultivačním médiu nemá zesílení expresse *ABP1* v buňkách BY-2 žádný měřitelný vliv na dělení buněk nebo na jejich prodlužování. Pěstování buněk se zesílenou expresí ABP1 v médiu s pětinásobně zvýšenou koncentrací 2,4-D vedlo k mírnému zvýšení buněčné elongace. Buňky s potlačenou expresí *ABP1* nejsou schopné prodlužování a intenzita buněčného dělení je výrazně zpomalena. ABP1 je tedy důležitý pro řízení buněčného cyklu a je nezbytný pro kontrolu rovnováhy mezi buněčným dělením a buněčnou expanzí. ABP1 je lokalizován v endoplazmatickém retikulu u buněk kultivovaných ve standardním médiu obsahujícím 2,4_D, ale pokud byly buňky vystaveny IAA namísto 2,4-D, ABP1 protein se objevil i na plazmatické membráně.

2) ABP1 reguluje transport auxinu mezi buňkami. Buňky se zesílenou expresí *ABP1* byly méně citlivé k inhibitoru aktivity přenašečů auxinu z buňky NPA, ale žádný rozdíl nebyl pozorován v případě použití PBA, inhibitoru dynamiky aktinu. Zesílení exprese *ABP1* za současně zesílené exprese transportérů auxinu typu PIN lokalizovaných na plasmatické membráně vedlo k potlačení fenotypu odpovídajícího auxinovému hladovění a mělo za následeik snížení exportu auxinu zprostředkovaného PINy. Tyto výsledky naznačují, že ABP1 působí na aktivní transport auxinů z buňky.

3) ABP1 zasahuje do procesů transportu váčků zajišťujících dynamiku přenašečů z rodiny PIN. Pomocí metody FRAP bylo zjištěno, že ABP1 zpomaluje dynamiku PIN1-GFP na plazmatické membráně. S použitím inhibitoru anterográdního transportu váčků (BFA) a inhibitoru endocytózy (Tyrphostin A23) bylo ukázáno, že účinek ABP1 je nezávislý na anterográdním transportu váčků, ale že ABP1 podporuje endocytózu PINů. Zesílená exprese

ABP1 vedla k ovlivnění aktivity fosfolipáz, které zprostředkovávají regulaci transportu váčků s proteiny PIN.

4) ABP1 je zapojeno v procesech vnitrobuněčného řízení hladin auxinu a jeho metabolismu. Za přítomnosti IAA v kultivačním médiu byla zvýšená exprese ABP1 schopna zabránit účinkům zesílené exprese na membráně endoplasmatického retikula umístěného transportéru *At*PIN5, a přispěla k ochraně volné IAA před konjugací. ABP1 tedy přispívá k udržení homeostáze auxinu uvnitř buněk tím, že zabraňuje PIN5-dependentním metabolickým změnám volné IAA.

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9 List of publications

Paper in impacted journal:

Petrášek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertová D, Wisniewska J, Tadele Z, Kubeš M, **Čovanová M**, Dhonukshe P, Skůpa P, Benková E, Perry L, Křeček P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C, Zažímalová E, Friml J: PIN proteins perform a rate-limiting function in cellular auxin efflux. SCIENCE 312: 914-918, 2006. ISI IF 2009: 29.747, cited 164x WOS, 165x SCOPUS

My contribution to this publication was the transformation of tobacco cell lines and their characterization , including the GVG-PIN7 line. I was also participating in labelled-auxin-accumulation assays and phenotypic studies.

The GVG-PIN7 line was then used as the source for double transformants carrying the tobacco *ABP1* gene. These double transformants were used in my further work and the results of experiments involving this line form part of the paper "Čovanová et al.: ABP1 negatively regulates PIN-dependent auxin transport" submitted to Plant Cell.

Manuscript in submission:

Čovanová M, Petrášek J, Sauer M, Friml J, Zažímalová E: ABP1 negatively regulates PIN-dependent auxin transport. Submitted to Plant Cell, 2010.

Other contributions:

Petrášek J, Seifertová D, Perry L, Skůpa P, **Čovanová M**, Zažímalová E: Expression of AtPINs in tobacco cells increases auxin efflux and induces phenotype changes typical for auxin depletion. Biol Plant 49 (Suppl): S10, 2005.

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Čovanová M, Petrášek J, Zažímalová E: Protoplast swelling as a measure of auxin action in BY-2 tobacco cell culture. Bulletin ČSEBR I: p. 54, 2006 (4^{th} Methods in Plant Science), Srní, Česká republika, October 1- 4, 2006).

Laňková M, Petrášek J, Perry L, **Čovanová M**, Zažímalová E: Characterization of a PaLAX1 permease as a presumptive auxin influx carrier. 14th International Workshop on Plant Membrane Biology, Valencia, Spain, June 26-30, 2007, Book of Abstracts, P7-1.

Skůpa P, Mravec J, **Čovanová M**, Petrášek J, Smetana O, Zažímalová E.: Characterization of tobacco cell lines transformed with the AtPIN5 gene from the auxin efflux carrier family of Arabidopsis. Comparative Biochemistry and Physiology part A 150, S194, 2008 (Society for Experimental Biology Annual Main Meeting, Marseille, France, July 6 - 10, 2008).

Čovanová M, Skůpa P, Petrášek J, Zažímalová E: Role of the Auxin binding protein 1 (ABP1) in intracellular auxin management. 3rd Symposium Auxins and Cytokinins in Plant Development, Prague, Czech Republic, July 10-14, 2009, Book of Abstracts, p. 72.

Čovanová M, Kubeš M, Petrášek J, Zažímalová E: Fluorescence Recovery After Photo-bleaching (FRAP) in Plant Cells. Bulletin ČSEBR I.: p. 47, 2009 (5th Methods in Plant Science, Malenovice, Czech Republic, October 11 – 14, 2009).

Čovanová M, Sauer M, Löfke C, Teichmann T, Friml J, Petrášek J, Zažímalová E: ABP1 mediates feed-back regulation of PIN-dependent auxin transport. Lecture selected from abstracts, Keystone symposia, Receptors and Signaling in Plant Development and Biotic Interactions, Tahoe City, California, USA, March 14-19, 2010. Book of Abstracts, p. 68.

10 Curriculum vitae

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Education

1998-2003 Czech Agricultural University, Institute of Tropical and Subtropical Agriculture, Prague, Czech Republic

2003 Degree in Agriculture (Ing., equivalent of Mgr.)

2005-2010 Postgraduate studies at the Faculty of Science, Charles University in Prague, Department of Experimental Biology of Plants, experimental work for the Dissertation thesis performed at the Academy of Sciences of the Czech Republic, Institute of Experimental Botany; Laboratory of Hormonal Regulations of Plants.

Research experience

2005-2010 Postgraduate student at Academy of Sciences of the Czech Republic, Institute of Experimental Botany; Laboratory of Hormonal Regulations of Plants.

2005 Internship at Biocenter Klein Flottbek, Department of Biology, University of Hamburg, November 21-December2, 2005

2001 Studies at Polytechnic University of Valencia, Spain, ERASMUS programme

Other experience:

Referee for scientific journals (Biologia Plantarum, South African Journal of Botany; both Springer/Kluwer)

4th Methods in Plant Science (4. Metodické dny), Metodické dny, Srní, Česká republika,

XIV. International Workshop, Plant Membrane Biology, Valencia, Spain, June 26-30, 2007

Auxins and Cytokinins in Plant Development, 3rd international symposium, Prague, Czech Republic, July 10 – 14, 2009

5th Methods in Plant Science (5. Metodické dny), Malenovice, Czech Republic, October 11 – 14, 2009

Keystone Symposia, Receptors and Signaling in Plant Development and Biotic Interactions, Tahoe City, California, USA, March 14 -19, 2010, lecture selected from abstracts