II.2 The BY-2 Cell Line as a Tool to Study Auxin Transport

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1 Introduction

Auxin transport plays a key role in the regulation of plant growth and development. Either it runs in apoplast by mass flow in the phloem together with other metabolites and/or there exists a parallel, cell-to-cell, strictly directional, carrier-mediated active transport. The major contribution to our understanding of its physiology as well as molecular background comes from studies *in planta*. However, during the last ten years, tobacco cell lines such as BY-2 have provided a major impetus for precise analysis of the machinery performing auxin flow across cell membranes at the cellular level. In this chapter recent knowledge about the molecular mechanism of auxin transport is summarized, and the data are discussed in the context of recent findings concerning the role of directional cell-to-cell transport of auxin in plant development. The results obtained using BY-2 as well as other tobacco cell lines highlight the advantages of these models in studies of auxin-regulated processes such as cell division, elongation and establishment of cell polarity.

2 Present State of the Art of Cell-to-Cell Transport of Auxins

Together with the processes of auxin biosynthesis, conjugation and degradation, unidirectional cell-to-cell transport of auxin (indole-3-acetic acid, IAA) plays a crucial role in the regulation of growth and development of plants (Blakeslee et al. 2005; Friml and Wisniewska 2005; Woodward and Bartel 2005). One of the most important features of auxin transport in the symplast is that it is unequivocally polar. The explanation of its polarity was simultaneously proposed by Rubery and Sheldrake (1974) and Raven (1975) and called the chemiosmotic polar diffusion model (Goldsmith 1977). According to this model, undissociated, a lipophilic form of native auxin molecule (IAA) can easily enter the cell cytoplasm from slightly acidic extracellular environment (pH 5.5) by passive diffusion. Since the pH of cytoplasm is more alkaline

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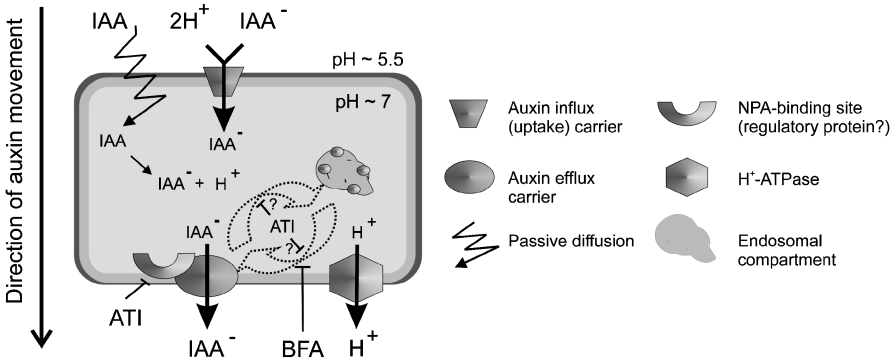

Fig. 1. Simplified scheme of auxin (IAA) transport at the cellular level. Both passive diffusion and specific auxin influx (uptake) and efflux carriers are involved in the transport of the IAA molecule and its dissociated form, auxin anion (IAA^-). In contrast to quite easy diffusion of relatively lipophilic molecules of IAA across the PM, hydrophilic IAA^- anions can be transported only actively via carriers. One of the auxin transport inhibitors (ATIs), 1-naphthylphthalamic acid (NPA), has been proposed to act via binding to the complex of auxin efflux carrier. Moreover, ATIs may have broader effect on protein trafficking processes (Muday et al. 2003). The fungal toxin brefeldin A (BFA) influences polar auxin transport by disturbing Golgi-mediated and actin-dependent vesicle transport of putative auxin efflux carriers from the intracellular space to the PM (Geldner et al. 2001, 2003)

(pH 7), IAA molecules dissociate and the resulting hydrophilic auxin anions (IAA^-) are trapped in the cytosol. The exit or uptake of IAA^- was proposed to be assisted by the auxin efflux and influx proteins, respectively, and the polarity of auxin transport was explained by their asymmetric distribution on the plasma membrane (PM) of the cell (Fig. 1).

In the last decade, genes encoding putative auxin uptake (influx) and efflux carriers have been identified in *Arabidopsis* and other species (Morris 2000; Muday and DeLong 2001; Friml and Palme 2002; Morris et al. 2004; Paponov et al. 2005). Since the intracellular concentration of auxin is, among other things, critical for the setting of various developmental programmes, it is obvious that oriented transport of auxin plays an important role in the morphoregulatory and pattern formation processes such as embryogenesis (Friml et al. 2003), root development (Friml et al. 2002a; Blilou et al. 2005; reviewed in Teale et al. 2005), organ formation (Benková et al. 2003), vascular differentiation (Mattsson et al. 2003), tropisms (Friml et al. 2002b) and phyllotaxis (Reinhardt et al. 2003).

3 Auxin Transport Studies in Planta

3.1 Auxin Transport Assays

It has always been challenging to follow the auxin distribution in plants and to understand how it is established and maintained. Besides modern instrumental techniques for the quantification of endogenous auxin content (Ljung et al. 2005) and non-invasive tracking of the expression of auxin-responding reporter genes (Ulmasov et al. 1997), the direct measurement of radiolabelled auxin distribution has been one of the most frequently used approaches in studies of auxin transport (Goldsmith 1977). Upon application of labelled auxin to one end of a tissue segment, auxin movement is usually followed by measurement of the quantity of transported radiolabel. Although this approach was recently optimized for the whole *Arabidopsis* seedling (Geisler et al. 2003) it is not applicable for the measurement of auxin transport at the cellular level.

3.2 Inhibitors of Polar Auxin Transport

One of the most fruitful approaches in studying the auxin transport machinery is the application of various inhibitors. The most widely used inhibitor of auxin efflux is 1-naphthylphthalamic acid (NPA), which belongs to a group of inhibitors known as phytotropins (Rubery 1990). The application of NPA to plant tissues results typically in an increase in auxin accumulation, presumably due to the inhibition of auxin efflux activity (Morris et al. 2004). Detailed knowledge about the mechanism, by which NPA and other phytotropins inhibit auxin efflux, is still lacking. NPA probably binds to a specific high affinity NPA-binding protein (NBP) located on the cytoplasmic face of the PM (Sussman and Gardner 1980), where it is associated with actin cytoskeleton (Cox and Muday 1994; Dixon et al. 1996; Butler et al. 1998). Recent evidence suggests that NBPs are aminopeptidases acting in cooperation with flavonoids, which are known as naturally occurring regulators of auxin efflux (Murphy et al. 2002). In addition, a more general, inhibitory effect of phytotropins on endocytotic processes was reported (Geldner et al. 2001).

Another set of results that helped the understanding of auxin transport machinery comes from studies using fungal toxin brefeldin A (BFA), the inhibitor of Golgi-mediated vesicle trafficking and endosomal recycling. BFA inhibits auxin efflux activity in zucchini hypocotyls (Morris and Robinson 1998) and blocks auxin transport through the tissue (Robinson et al. 1999). Since BFA treatment effectively changes the proportion of proteins localized at the PM and in the endosomal space (reviewed in Geldner 2004), it is an ideal tool for studying constitutive cycling of both putative auxin efflux and uptake carriers.

3.3 Genetic and Molecular Characterization of Components of the Auxin Transport Machinery

Recent research using the model plant *Arabidopsis thaliana* led to the identification of proteins involved in the auxin transport machinery. These include promising candidates for both auxin uptake (influx) carrier, AUX1/LAXs (amino-acid permease-like proteins), and regulators of auxin efflux, PINs (PIN-formed, plant-specific PM proteins) and MDR/PGPs (multidrug-resistance-like/P-glycoproteins) (see Morris et al. 2004; Blakeslee et al. 2005; Friml and Wisniewska 2005). It seems that PIN proteins establish the direction of auxin flux by their asymmetric distribution at the PM. They may form complexes with other regulatory proteins and MDR/PGPs may stabilize these complexes (Blakeslee et al. 2005).

The correct positioning of the auxin efflux complex seems to be assisted by the actin cytoskeleton. The application of actin drugs resulted in reduced polar auxin transport in maize coleoptiles (Cande et al. 1973) and in zucchini hypocotyls (Butler et al. 1998). Moreover, using BFA, it was shown that PIN proteins might undergo constitutive cycling between PM and the endosomal space, as indirectly suggested by Robinson et al. (1999), and that this process is actin-dependent (Geldner et al. 2001). It was shown that the mutation in *Arabidopsis* myosin VI led to the inhibition of basipetal auxin transport (Holweg and Nick 2004). All these observations strongly suggest that actin filaments are involved in both intracellular traffic of PINs and their correct targeting to the proper domains at the PM. The concept of trafficking and proper localization as well as function of components of the auxin efflux carrier complex has been proposed (summarized in Muday et al. 2003). In contrast to this, the mechanism(s) underlying the constitutive cycling of proteins in plants is still poorly understood (Murphy et al. 2005). Constitutive cycling of PINs is regulated by GNOM, one of the exchange factors for ADP-ribosylation factor-type GTPases (ARF-GEFs; Geldner et al. 2003, 2004) and other ARFs might also contribute to the trafficking of PINs (Xu and Scheres 2005). Interestingly, the regulation of endocytosis-dependent cycling of proteins in plant cells was shown to be regulated by auxin itself (Paciorek et al. 2005), suggesting a new mechanism of auxin action in plant cells.

4 Auxin Transport Studies in Simplified Models

4.1 Transport of Auxins in Suspension-Cultured Cells

Since it is difficult to study the biochemical aspects of auxin transport by measurements at the whole-plant and organ level (see above), plant cells cultured in liquid medium represent a valuable alternative. The accumulation of auxin can be measured in time intervals after the direct addition of radiolabelled auxin

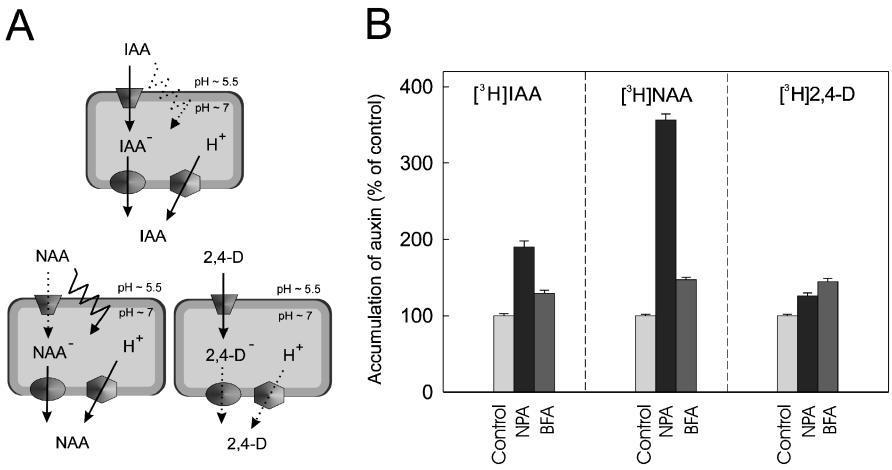

Fig. 2. Differences in the translocation of IAA and two synthetic auxins NAA and 2,4-D in tobacco cell lines. **A** Auxins are translocated across PM according to their relative lipophilicity (NAA > IAA > 2, 4-D). As shown by careful measuring of diffusion and carrier-mediated transport of NAA, IAA and 2,4-D in tobacco suspension-cultured Xanthi XHFD8 cells by Delbarre et al. (1996), the accumulation level of NAA is controlled mainly by efflux carrier activity, while the accumulation of 2,4-D is determined by the activity of an uptake carrier. Both uptake and efflux carriers, as well as passive diffusion, contribute to the accumulation of IAA. Refer to Fig. 1 for *symbol* legends. **B** The accumulation of all three types of auxins (IAA, NAA, 2,4-D) is increased upon application of inhibitors NPA or BFA, thus reflecting disturbed auxin efflux machinery in BY-2 cells (Petrášek et al. 2005)

into the cell suspension. Indeed, the most important experiments, on which the chemiosmotic hypothesis of IAA transport was based, were performed using auxin-autonomous suspensions of crown gall cells of Boston Ivy (Rubery and Sheldrake 1974), where the time of addition as well as concentrations of auxin transport inhibitors or auxin itself could be readily controlled.

The first characterization of auxin transport mechanisms at the cellular level was reported by Delbarre et al. (1994, 1996) in mesophyll protoplasts and cell suspension from tobacco cv. Xanthi XHFD8, respectively. As depicted on Fig. 2A, Delbarre et al. proposed a simple method to describe the role of passive diffusion and active carrier-mediated processes in the transport of three of the most common auxins, namely native IAA, synthetic naphthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). The same cell suspension was used for the characterization of a new class of inhibitors of auxin influx (Imhoff et al. 2000) as well as for the study of phosphorylation/dephosphorylation of proteins of the auxin efflux complex (Delbarre et al. 1998). One such regulatory protein, PINOID kinase, was recently shown to regulate polar targeting of PIN proteins in *Arabidopsis* (Friml et al. 2004).

In contrast to cell suspensions of *Arabidopsis* (which tend to form large cell clusters), the major advantage of intensively dividing cell cultures of tobacco

is that the effects on cell morphology of various inhibitors as well as of auxin itself can be observed in parallel with the measurements of auxin accumulation. Since tobacco cell lines of a good quality are completely friable, it is possible to express all measured data as an equivalent of cell number. During the period of cell division, such high-quality tobacco cell lines usually form polar cell files instead of cell clusters. Based on the studies using tobacco cell line VBI-0 (*Nicotiana tabacum* L., cv. Virginia Bright Italia; Opatrný and Opatrná 1976), the inhibition of auxin transport by NPA and the consequent rise in the internal auxin level delayed the onset of cell division and disrupted its polarity (Petrášek et al. 2002). Mathematical modelling suggested that NPA possibly disturbs the gradient in auxin concentration along the cell file (Campanoni and Nick 2003). This effect might be mediated by the actin cytoskeleton, as shown by Holweg et al. (2003), using inhibitors of myosin action. In spite of the fact that the auxin-autonomous cell line VBI-I1 containing spherical cells was derived (Campanoni et al. 2001) from 'parental' VBI-0 line, VBI-0 itself is routinely maintained on both NAA and 2,4-D. These two auxins may control cell division and cell elongation by different pathways (Campanoni and Nick 2005); therefore, the BY-2 cell line is a better model in this respect, as it is only 2,4-D-dependent.

4.2 Transport of Auxins in BY-2 Cells

The potential of simultaneous measurements of intracellular auxin accumulation and tracking of changes in various cell structures, together with directed transgenosis, makes tobacco BY-2 cells an invaluable tool for the study of auxin transport at the cellular level (Petrášek et al. 2003; Zažímalová et al. 2003) as well as for the characterization in vivo of functions of the proteins involved. BY-2 cells respond to the addition of inhibitors NPA or BFA by an immediate rise in the accumulation of IAA, NAA and 2,4-D (Fig. 2B). The transport of these auxins occurs in similar ways as suggested for tobacco Xanthi XHFD8 cells (Delbarre et al. 1996; Fig. 2A). Both the kinetics of NAA accumulation (Fig. 3A) and the arrangement of the cytoskeleton reflect differential sensitivity of BY-2 cells towards BFA and NPA (see Petrášek et al. 2003 for details).

To test the proposed role of PIN proteins in the regulation of auxin transport, BY-2 cells were transformed with *PIN1*, *PIN4*, *PIN6* and *PIN7* genes from *Arabidopsis thaliana*. For all tested PINs, their strong inducible overexpression resulted in a decrease in auxin accumulation (Fig. 3B; Skùpa et al. 2005), followed by remarkable changes in cell morphology (Fig. 3D, E, F). Similar changes have been reported previously to be typical of the response to auxin depletion (Sakai et al. 2004). Moreover, the fact that NPA, an inhibitor of auxin efflux, was capable of preventing all observed "auxin-depletion"-induced changes (Fig. 3G) strongly points to the modification in auxin transport (Petrášek et al. 2005). In vivo studies using BY-2 cells expressing *Arabidopsis* PIN1 protein fused to GFP revealed its predominant localization at transversal PMs, although

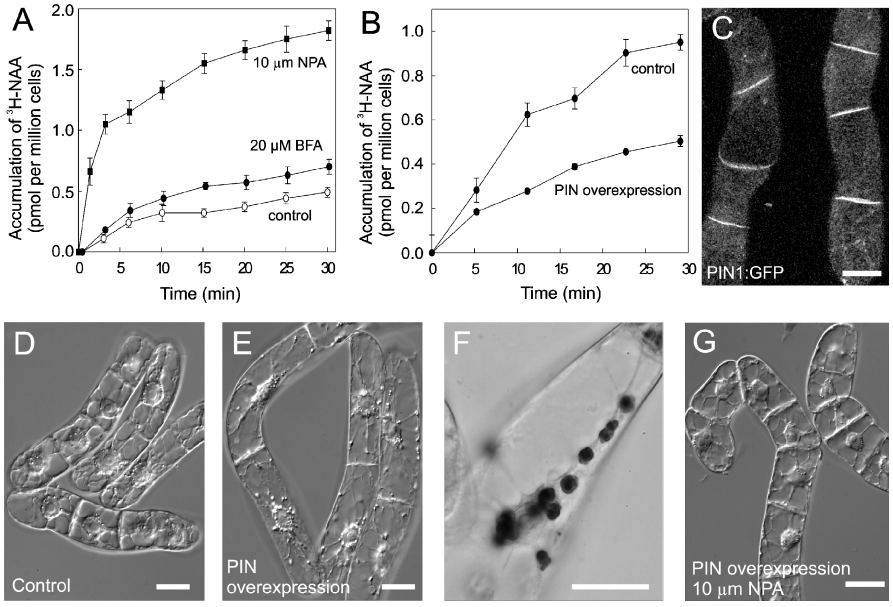

Fig. 3. Treatments with auxin efflux inhibitors or overexpression of putative auxin efflux carriers result in changed levels of endogenous auxin and distinct cell morphology in BY-2 cells. **A** Accumulation kinetics reflecting the increase in accumulation of auxin (NAA) upon application of inhibitors of auxin efflux, BFA and NPA (Petrášek et al. 2003). *Error bars* represent SEs of the mean (n = 4). **B** Decrease in accumulation of auxin upon PIN protein overexpression reflecting enhanced auxin efflux (Skùpa et al. 2005). *Error bars* represent SEs of the mean (n = 4). **C** Two-day-old BY-2 cells stably expressing *Arabidopsis* PIN1 protein in translation fusion with GFP. Merged five optical sections (each section 1 μm) through the cortical cytoplasm. The localization of PINs in BY-2 cells at transversal PMs suggests preferential direction of auxin flow. **D–G** Three-day-long overexpression of PIN proteins under strong promoter results in the cessation of cell division (**E**) and formation of starch-containing amyloplasts (Lugol staining) (**F**). All changes are rescued by simultaneous treatment with 10 μM NPA (**G**) to control-like situation (**D**), suggesting the inhibition of overexpressed PIN proteins (Petrášek et al. 2005). *Scale bars* 20 μm

a proportion of fusion protein was localized along longitudinal PMs and in the cortical cytoplasm (Fig. 3C). Thus, in the BY-2 cell line the distribution of PIN1-GFP in cell files resembles the PIN distribution pattern in the cells of the root elongation zone of *Arabidopsis thaliana*.

It was recently shown (Paciorek et al. 2005), using *Arabidopsis thaliana* plants as well as both BY-2 and VBI-0 tobacco cells, that auxins can inhibit the endocytotic step of the constitutive cycling of PM proteins including PIP2 aquaporin, PM-ATPase and PINs. According to these results, auxin increases levels of PINs at the PM and concomitantly promotes its own efflux from cells. This finding implies a novel mode of auxin action, similar to some animal hormones and consistent with pleiotropic physiological effects of auxin on plant cells.

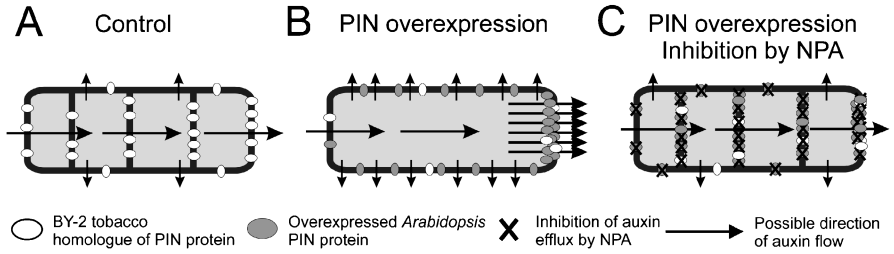

Fig. 4. Schematic diagrams summarizing the effects of overexpression of the PIN proteins in BY-2 cells. **A** Control, exponentially growing cells expressing their endogenous tobacco efflux carriers. According to BLAST search in the EST database of Matsuoka et al. (2004), EST n. 3440f1 is the most promising candidate for an Nt*PIN* gene. **B** Strong overexpression of *Arabidopsis* PIN protein stimulates auxin efflux. As a consequence, cells display typical auxin-starvation symptoms (Winicur et al. 1998; Sakai et al. 2004), such as inhibition of cell division, stimulation of amyloplast differentiation and marked cell elongation. **C** Upon inhibition of a proportion of endogenous as well as overexpressed auxin efflux carriers by NPA, cell division is restored to the control-like situation (Petrášek et al. 2005)

Altogether, our observations, summarized in Fig. 4, reveal that changes in BY-2 cell development are related to auxin transport and – from the point of view of molecular mechanisms of auxin transport – they strongly support the idea that PIN proteins are the rate-limiting components of the auxin efflux machinery, and probably the auxin efflux catalysts themselves.

5 Concluding Remarks and Future Prospects

In conclusion, the BY-2 tobacco cell line together with a few other well-defined tobacco cell lines, can serve as an invaluable alternative experimental tool for auxin transport studies complementary to *Arabidopsis* plants. In our laboratory, the collection of BY-2 cells, transformed with components of both auxin influx (Laòková et al. 2005) and efflux machinery (see above), is maintained and constantly extended. In future, these experimental materials may be useful for studies of the composition of the auxin transport machinery and quantitative aspects of its action, as well as for the characterization of the dynamics of proteins that play a role in the transport of auxin in vivo using fluorescent tags. It may also contribute to discovering the role of both auxin influx and efflux in the establishment of auxin levels inside the cell and their relation to the regulation of cell division, cell elongation and establishment and/or maintenance of cell polarity.

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