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THE DYNAMICS OF AUXIN TRANSPORT IN TOBACCO CELLS

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Summary. The polar transport of auxins plays a key role in the regulation of growth and development in plants. The experimental evidence indicates that the polarity of auxin transport through cells and tissues probably results from the asymmetrical distribution of auxin efflux carriers in the plasma membrane. A substantial amount of molecular and cytological data about the polar auxin transport machinery comes from studies at the tissue level on *Arabidopsis thaliana* and its mutants. However, there is a need for some alternative experimental system that would enable us to study auxin transport from biochemical and cytological points of view in more detailed way. Cell suspension cultures provide good model systems in which to study biochemical and cytological aspects of auxin transport and to investigate the responses of transport to various inhibitors at the level of a single cell and its compartments. This mini-review summarises the present state-of-the-art in the investigation of the auxin transport machinery in tobacco cell cultures. The specificity of auxin efflux carriers is described, together with the effects on auxin transport and on the arrangement of the cytoskeleton and of the endoplasmic reticulum (ER), of the polar auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) and the vesicle-trafficking inhibitor brefeldin A (BFA). The data emphasise the importance of actin filaments and possibly also of the ER in vesicle-mediated trafficking of proteins and in the cycling of the auxin efflux catalyst. The data also imply that different cellular mechanisms are involved in the actions of NPA and BFA. Contrary to the recent suggestion that auxin transport inhibitors influence auxin efflux by generally interfering with membrane trafficking processes, in tobacco cells NPA seems to act specifically

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on auxin efflux. The available data suggest that an NPA-binding regulatory protein may anchor the auxin efflux catalyst to the actin filaments and may be involved in directing its proper localisation to specific regions of the plasma membrane.

Key words: Auxin carrier, Auxin transport, Brefeldin A, Cytoskeleton, 1-N-Naphthylphthalamic acid, *Nicotiana tabacum* L.

Abbreviations: AFs – actin filaments; BFA – brefeldin A; 2,4-D – 2,4-dichlorophenoxyacetic acid; ER – endoplasmic reticulum; IAA – indole-3-acetic acid; MTs – microtubules; NAA – 1-naphthaleneacetic acid; NBP – NPA-binding protein; NPA – 1-N-naphthylphthalamic acid; PM – plasma membrane;

Introduction

In addition to the genetically determined programme, plant growth and development is controlled by both chemical and environmental stimuli. Plant growth regulators, including the native phytohormones, are typical representatives of the chemical ones. The fact that plant growth regulators significantly affect plant development has long been recognised and documented from the level of the single cell to that of the whole plant. In plant cells most of the basic physiological processes such as cell division, cell elongation, polarity, and differentiation are controlled, amongst other factors, by phytohormones – above all by auxins and cytokinins (Skoog and Miller, 1957; Kamínek, 1992; Cleland, 1995; Davies, 1995; Chen, 1997; D'Agostino and Kieber, 1999; Berleth et al., 2000; Pasternak et al., 2000; Haberer and Kieber, 2002).

Any plant cell possesses mechanisms precisely to regulate internal levels of important substances, including phytohormones and phytohormone-like compounds. Since the internal concentration of any particular compound results from the partial contributions of several metabolic and transport events, control of this concentration is a rather complex process. Metabolic regulation involves biosynthesis, conjugation and degradation of the active compound. At the transport level, the concentration of the active compound at sites of action within the cell is influenced by processes that affect both the passive and active uptake and efflux of the compound into and/or out of the cell (i.e. the exchange between 'outer' and 'inner' environment), as well as by its transport into and out of individual cell compartments.

The rate of movement of any compound across the plasma membrane (PM) and its resulting internal net accumulation is affected by the relative lipophilic or hydrophilic nature of its molecule(s). Generally, transmembrane transport can proceed in both passive (diffusive) and carrier-driven manners. Therefore the translocation of phytohormones and phytohormone-like substances depends not only on the prevailing

ionic form of their molecules (which is a function of the pH in the environment or in a particular cell compartment), but also on the presence/absence of compatible carrier(s).

Polar auxin transport – structures and mechanisms

Auxins seem to be the only group of plant hormones to undergo both an active, polar transport from cell to cell through whole plants and plant parts, and long-distance translocation in the xylem and phloem translocation systems (Hopkins, 1995; Baker, 2000a, b). Polar transport of auxin was first demonstrated in late twenties (Went, 1928). Its polarity was later explained in general (Rubery and Sheldrake, 1974; Raven, 1975) by the different relative permeabilities of opposite ends of cells to dissociated and undissociated forms of molecules of indole-3-acetic acid. This differential permeability was shown later to result from an asymmetric distribution in the plasma membrane of specific auxin anion efflux carriers. The evidence for such a mechanism was discussed in detail by Goldsmith (1977), who termed it "chemiosmotic polar diffusion" (a shortened form of "proton and electron motive force (emf) dependent polar diffusion"; Goldsmith, 1977). It is now believed – on the basis of biochemical, physiological and molecular data – that an auxin-uptake (influx) carrier drives auxin translocation into the cell and that a different carrier system mediates auxin efflux. To different extents the activity of both carriers can be inhibited by several synthetic compounds, including phytotropins such as NPA (see reviews by Rubery 1990; Lomax et al., 1995; Bennett et al., 1998; Morris, 2000; Muday and DeLong, 2001). Application of NPA to root tissues of *Arabidopsis* results in blockage of the first transverse division of xylem pole pericycle cells; and lateral root development is arrested probably as a result of IAA accumulation in the root apex and a decrease of its level in basal tissues (Casimiro et al., 2001). 1-Naphthoxyacetic and 3-chloro-4-hydroxyphenylacetic acids are reported to inhibit auxin uptake (influx) carrier activity and both these compounds are able to disrupt root gravitropic responses and to mimic the auxin uptake (influx) carrier mutation *aux1* (Parry et al., 2001).

Inhibitors of protein synthesis have been shown to uncouple auxin efflux and the inhibition of efflux by NPA, without in the short term affecting either efflux itself or specific NPA binding (Morris et al., 1991). These observations led to the suggestion that unlike the auxin uptake carrier, which is probably a single protein, the efflux carrier is a complex multi-component system consisting of the efflux catalyst itself, an associated phytotropin-binding regulatory protein (the so-called NPA-binding protein, NBP) and, possibly, a third, rapidly turned over, protein that is required for the interaction between the NBP and the transport catalyst (Morris et al., 1991). Little is known about the mechanisms that result in the proposed asymmetric distribution of this multi-component system. There is some evidence that the cytoskeleton (in particular, microfilaments; Cox and Muday, 1994; Butler et al., 1998; Hu et al., 2000;

reviewed by Muday, 2000; Muday and DeLong, 2001; Luschnig, 2001; Muday and Murphy, 2002) and/or Golgi-mediated protein traffic may be involved in the establishment and maintenance of this distribution (Robinson et al., 1999; Geldner et al., 2001). The localisation of the auxin efflux carrier complex to specific regions of the PM is not static; the complex seems to cycle rapidly between the PM and an as yet unidentified endosomal compartment (Wilkinson and Morris, 1994; Robinson et al., 1999; Geldner et al., 2001). Based on studies of internalisation of the putative auxin efflux carrier (*PIN* gene product) and some other proteins in *Arabidopsis thaliana* roots, Geldner et al. (2001) suggested, that the inhibitors of auxin transport possess a more general role in the regulation of vesicle-mediated protein traffic to the plasma membrane.

By 1998, several genes coding for both auxin uptake and efflux carriers and for NPA-binding proteins had been characterised. Typical representatives of these genes identified in *Arabidopsis thaliana* included *AUX1* (coding for the auxin uptake carrier), the *PIN* gene family (coding for the putative auxin efflux carriers, and differentially expressed in different tissues and organs), and *TIR3/DOC1* (coding for a putative NBP; summarised in Bennett et al., 1998; Gälweiler et al., 1998; Morris, 2000; Friml and Palme, 2002). Gil et al. (2001) found that the *tir3* and *doc1* mutants were allelic and *TIR3/DOC1* was renamed *BIG* to reflect the extraordinary size of its protein product. The analogous mammalian protein is involved in vesicle trafficking. BIG protein is able to bind NPA, and itself binds to the actin filaments. This may imply that BIG is involved in the establishment of the asymmetric localisation of auxin efflux carriers (Muday and DeLong, 2001; Luschnig, 2001; Muday and Murphy, 2002).

Recently, other genes that encode potential auxin carriers have been identified from *Arabidopsis*. These include the *ANT1* gene that encodes an aromatic and neutral amino acid transporter and which may serve as an influx carrier for both IAA and 2,4-D (Chen et al., 2001). Several genes related to animal *multidrug resistance* (*MDR*) genes (a sub-group of the highly conserved ABC transporter gene family), have also been found in *Arabidopsis*. These encode transporters possessing very wide substrate specificity and which function in an energy-dependent manner. Two of the *Arabidopsis* proteins encoded by *MDR* genes, AtMDR1 and AtPGP1, have been shown recently to specifically bind NPA with high affinity and specificity, and may also transport auxins themselves across both the plasma membrane and intracellular membranes (Noh et al., 2001; reviewed by Luschnig, 2002). The present state of our knowledge of carriers and potential carrier for auxins is summarised in Fig. 1.

There is now an extensive and rapidly growing body of knowledge about the transport of auxin and new information on the topic appears almost every day. However, the data relate to such a diverse range of plant systems, often studied at different stages of their development, that it is difficult to extrapolate from one to another or to draw general conclusions. Up to now, there have been very few reports summarising physiological, biochemical, genetic and cytological data obtained from one experimental material and relating to a particular developmental process. Recent data, including

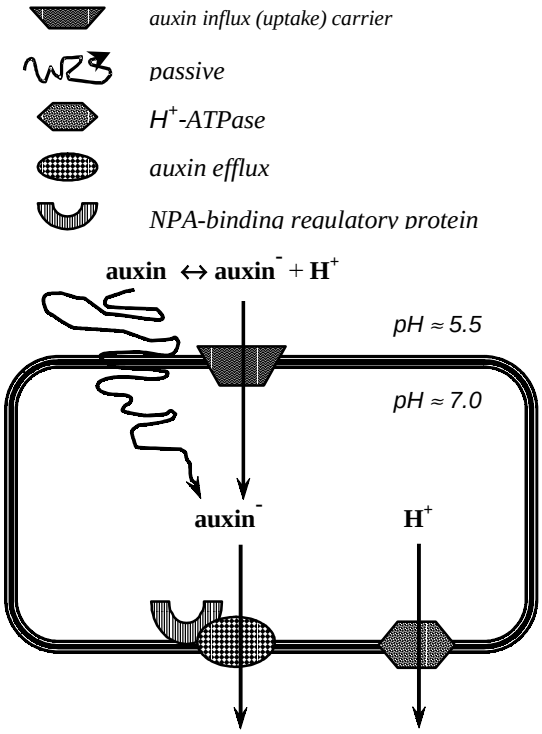

Fig. 1. The components of auxin transport across the plasma membrane of plant cells.

much valuable information at the molecular and genetic level, have been derived mainly from work on various tissues and/or organs, but primarily roots, of *Arabidopsis thaliana* (e.g. Parry et al., 2001; Friml et al., 2002a, b; summarised in Muday, 2001; Friml and Palme, 2002; Friml, 2003). This is, perhaps, not surprising given the predominant position of this plant as a model for molecular genetic investigations. Nevertheless, it is difficult to study the behaviour of individual cells from the biochemical and/or cytological point of view when they are situated within intact plant organs. Furthermore, the diversity of cell types within even the tiny roots of *Arabidopsis* make it almost impossible to investigate biochemical aspects of auxin transport at the cell level in a meaningful way, especially given the present level of sensitivity of the biochemical techniques available. This problem might be overcome by the development of a suitable cell suspension culture system in which individual cells separated easily, and particularly one in which the stages of cell development can be readily synchronised. So far, no well-established, good quality cell culture system possessing these characteristics has been derived from *Arabidopsis* tissues, and there is an urgent need for an alternative model system in which to conduct parallel studies of the cellular and biochemical aspects of polar auxin transport.

Some well-defined tobacco cell lines might furnish suitable alternative model cell systems. Some have characteristics (such as ease of culture, friability and near synchrony) that might allow them to be used in ways that would contribute significantly to our understanding of auxin transport and its regulation, especially in relation to the main processes of plant cell development, namely division, enlargement and the establishment and/or maintenance of cell polarity. Possible candidate cell lines include the facultatively auxin-dependent, cytokinin-autonomous cell strain BY-2 (*Nicotiana tabacum* L., cv. Bright Yellow 2; Nagata et al., 1992) and VBI-0 (*Nicotiana tabacum* L., cv. Virginia Bright Italia; Opatrný and Opatrná, 1976; Zažímalová et al., 1995). When cultivated in cell suspension culture under standard cultivation conditions, both cell strains exhibit a specific filamentous phenotype and a stable growth cycle (Fig. 2). Moreover, especially in the case of the VBI-0 cell line, the processes of cell division and elongation are well time-separated, thus allowing the study of both processes consecutively on the same cell population. As documented earlier, both VBI-0 and BY-2 strains are auxin-dependent (both 2,4-D and NAA for VBI-0; only 2,4-D for BY-2) and cytokinin-autotrophic. Their high spontaneous friability, good (VBI-0), or even extremely high (BY-2) growth (cell multiplication) rate and semi-synchronous cell division are advantageous for parallel cytological and biochemical analyses. The cells of these lines, particularly those of VBI-0, are relatively large (e.g. ca. 500 μm and 100 μm long in representative stationary phase cells of VBI-0 and BY-2, respectively – see Fig. 3). This makes such cultures a good model in which to visualise subcellular structures, including the cytoskeleton and various endomembrane structures (for re-

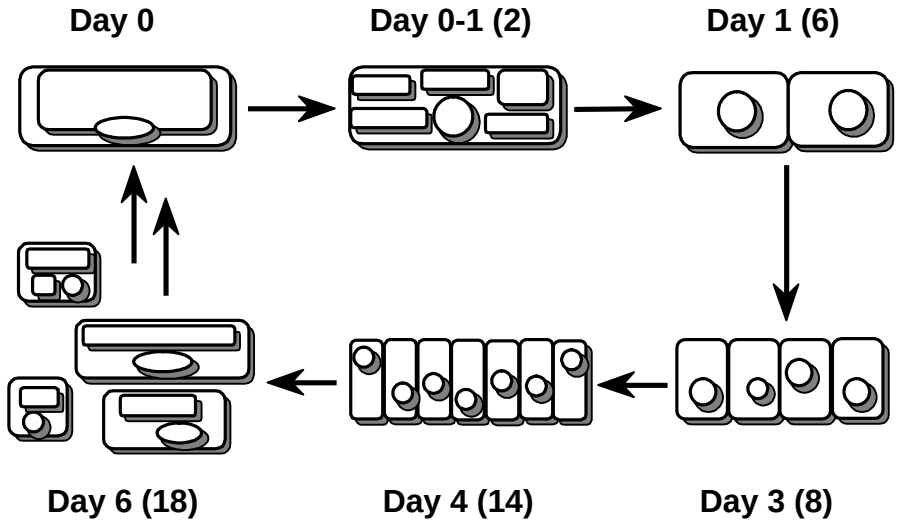

Fig. 2. Scheme of the growth cycle of suspension-cultured BY-2 and VBI-0 tobacco cells. The numbers represent the days after inoculation for BY-2 and VBI-0 (in brackets), respectively. Modified from Petrášek et al. (2002a).

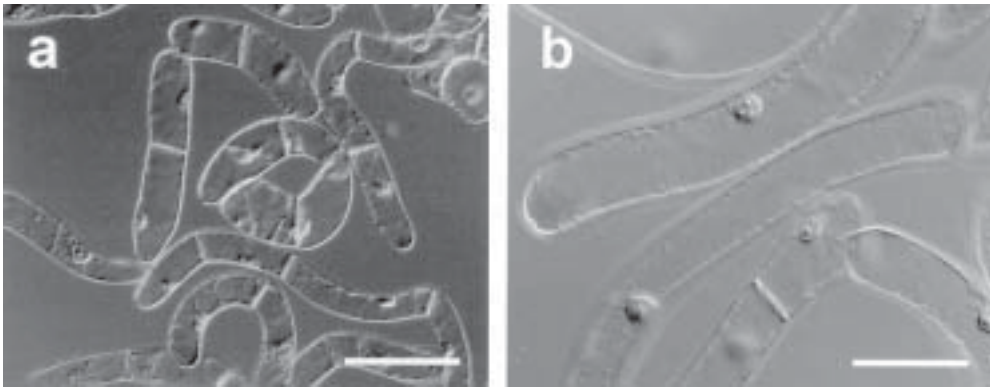

Fig. 3. Comparison of phenotypes of suspension-cultured BY-2 and VBI-0 tobacco cells in the stationary phase of growth. Nomarski differential interference contrast (DIC). **a**. BY-2 cells at day 7 of the subculture interval. **b**. VBI-0 cells at day 18 of the subculture interval. Scale bars = 100 μm.

views on BY-2 see Nagata et al., 1992; Kumagai and Hasezawa, 2001; for VBI-0 see Petrášek et al., 1998; Schwarzerová et al., 2002). Since when cultivated as batch cultures, both lines are very friable (i.e. they do not form cell clumps), any compound in the liquid cultivation medium is equally and very rapidly accessible for every cell in the suspension. This makes biochemical analyses of transmembrane transport (eg. the measurement of its kinetics, its concentration dependence, and the effects on it of inhibitors) more precise in cell suspensions than in whole organs or tissue segments.

Auxin carriers in tobacco cells

In contrast to the *PIN* and *AUX1* genes of *Arabidopsis,* nothing is known about the genes which encode putative auxin carriers in tobacco. The mechanism of auxin accumulation at the cell level in tobacco was described first by Delbarre et al. (1996) using cultured cells of *Nicotiana tabacum*, cv. Xanthi XHFD8. Using various radiolabelled auxins, the biochemical properties (kinetic properties and specificity) of both influx and efflux carriers were determined, and data was obtained on the interactions of carriers with potential inhibitors, including NPA and 2-naphthaleneacetic acid (2-NAA). Taken together, the results revealed that different auxins were translocated across cell membranes in different ways according to their relative lipophilicity (NAA > IAA > 2,4-D). The most lipophilic NAA molecules entered cells mainly by passive diffusion, but required the participation of efflux carriers for excretion from the cells (presumably as anions). In contrast the most hydrophilic auxin, 2,4-D, was taken up mostly via an influx carrier and then accumulated inside the cell because it was not a substrate for an auxin efflux carrier. The native auxin, IAA, was a good substrate for both influx and efflux carriers. On the basis of the different substrate specificities for NAA and 2,4-D of uptake and efflux carriers, Delbarre et al. (1996) proposed a simple method to distinguish between them and to determine their relative

activities. Transport (efflux) of NAA was a good indicator of efflux carrier activity, whilst in contrast, accumulation of 2,4-D was held to serve as a marker of auxin influx carrier activity. This approach has been used for identification of new auxin transport inhibitors of aryl and aryloxyalkylcarboxylic acid type (Imhoff et al., 2000). Delbarre et al. (1998) found that the activity of auxin transport in tobacco cells was probably regulated by phosphorylation/dephosphorylation. This was in agreement with the finding that *rcn1* ("**r**oots **c**url in **N**PA") mutant, which exhibited an impaired gravitropic response in the presence of NPA, could be phenocopied with a phosphatase inhibitor (Deruere et al., 1999). The corresponding *RCN1* gene was found to encode a subunit of protein phosphatase 2A (Garbers et al., 1996). Hypothetically, phosphatase could be a candidate for a third putative component of the auxin efflux carrier complex (cf. Morris et al., 1991). A partnering kinase could be represented by PINOID, which is a protein kinase, and *pinoid* mutants showed enhanced polar auxin transport (Benjamins et al., 2001).

It has been shown that net auxin accumulation in both suspension-cultured tobacco cells (Delbarre et al., 1998; Petrášek et al., 2002b) and zucchini segments (Wilkinson and Morris, 1994; Morris and Robinson, 1998) increased in the presence of inhibitors of vesicle trafficking such as monensin and brefeldin A (BFA). BFA is known to inhibit anterograde vesicle transport (see Nebenfûhr et al., 2002, for more information about BFA action). Consequently, BFA treatment would cause auxin efflux carrier complexes (or one or more of their individual components) to be retained in endosomal compartments and would reduce their traffic to, and action in, the PM. As a result, net auxin accumulation in the cell would rise. The observed effect of BFA on auxin accumulation in tobacco cells was in good agreement with the previously reported cycling of the auxin efflux carrier (the transport catalyst itself and/or components of auxin efflux carrier complex, see above). The sensitivity of various tobacco cells towards BFA was almost identical: 50% inhibition of auxin efflux was reached at ca. 3 μM BFA in cell suspensions of both *Nicotiana tabacum*, cv. Xanthi XHFD8 (Delbarre et al., 1998) and BY-2 (*Nicotiana tabacum*, cv. Bright Yellow-2; Petrášek et al., 2003). The dose-response curve for BFA treatments had an optimum at 10-30 μM BFA and decreased markedly at concentrations of BFA higher than 40 μM (Petrášek et al., 2003). The BFA concentrations having the highest effect on tobacco suspension-cultured cells are in good agreement with those used for BFA-induced internalisation of PIN proteins in *Arabidopsis* roots (Geldner et al., 2001), the finding reflecting the probably very similar sensitivity of plant cells towards this vesicle-trafficking inhibitor.

A number of synthetic compounds inhibit auxin efflux. Some of them, in particular the phytotropins (Katekar and Geissler, 1980; Rubery, 1990; so-called because they inhibit auxin-dependent tropic responses) seem to be very specific and efficient. NPA is a typical representative of this class of inhibitors. It binds to a specific NPA-binding regulatory protein (NBP, see above) and, in consequence, it inhibits the action of the auxin efflux catalyst. Indeed, the application of NPA led to an increase in net auxin

accumulation by tobacco cells (Delbarre et al., 1996; Petrášek et al., 2002b; Petrášek et al., 2003). Although NPA mainly inhibited the auxin efflux carrier, to a much lesser extent it also inhibited the influx carrier (Delbarre et al., 1996). The sensitivity of different tobacco cells towards NPA was not the same. The half-inhibition of auxin efflux carriers occurred at concentrations of NPA varying from 0.3–0.5 μM (Delbarre et al., 1996) to as low as ca. 0.05 μM (Petrášek et al., 2003). There was a significant decrease of this inhibition at concentrations of NPA higher than 30 μM (Petrášek et al., 2003). Comparison of half inhibition values for BFA (3 μM) and NPA (0.05 μM), optimum values (10–30 μM and 0.3–1 μM, for BFA and NPA, respectively) and the decrease of inhibition at concentrations above 40 μM and 100 μM (BFA and NPA, respectively) results in bell-shaped dose-response curves for both BFA and NPA (Fig. 4). According to this data, the tobacco cells are much more sensitive towards NPA than towards BFA.

It has been suggested recently that the synthetic inhibitors of auxin transport act generally to impair vesicle-mediated traffic of proteins to PM (Geldner et al., 2001). This suggestion implies that the action of "auxin transport inhibitors" is not in fact auxin transport-specific, but is related more to the mode of action of vesicle-trafficking inhibitors. However, as summarised above and in Petrášek et al. (2003), detailed analysis of the kinetics and concentration dependence of auxin transport inhibition by NPA in tobacco cell suspensions does not support this suggestion. A number of other observations are not readily explained by the suggestion. Firstly, in tobacco cells NPA

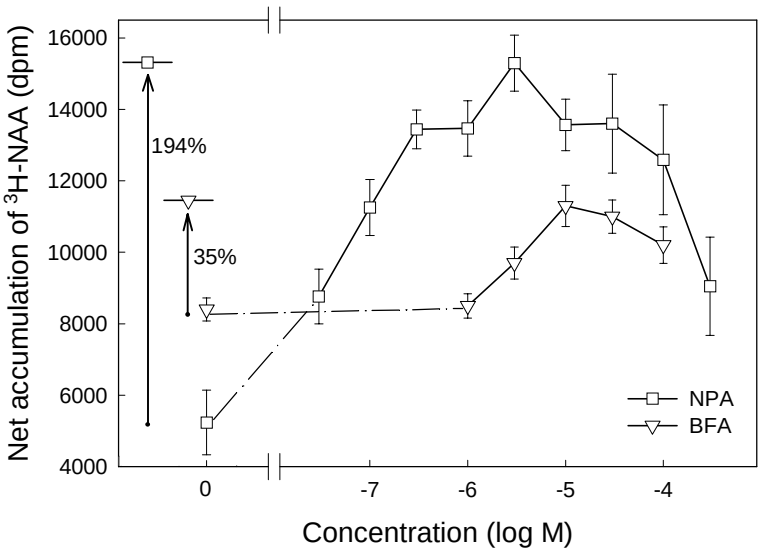

Fig. 4. The effect of concentrations of brefeldin A (BFA) and 1-N-naphthylphthalamic acid (NPA) on the net accumulation of ^{3}H-NAA (2 nM) in 2-day-old BY-2 cells. Bars represent standard deviations (n=3). Vertical arrows show the maximum stimulation by NPA and BFA compared to controls (the increases are shown as percentages of the control values; controls = 100%).

inhibited auxin efflux (i.e. stimulated net intracellular auxin accumulation) without a measurable lag. Secondly, the stimulation of auxin accumulation (i.e. inhibition of efflux) is saturated by concentrations of NPA some two orders of magnitude lower than those reported by Geldner et al. (2001) to be necessary to inhibit the putative auxin efflux carrier cycling. Thirdly, most of the data presented by Geldner et al. (2001) relate to TIBA. However, for reasons outlined in Petrášek et al. (2003), TIBA is not a good representative of specific polar auxin transport inhibitors and does not belong to the group of phytotropins (Katekar and Geissler, 1980; Depta et al., 1983; Depta and Rubery, 1984). Furthermore, at the concentration used by Geldner et al. (2001; 25 μM TIBA) there is a strong likelihood that TIBA may have caused cytoplasmic acidification (see Depta and Rubery, 1984). A further interesting and potentially important observation arises from the detailed measurements made on tobacco cells: while NPA at optimal concentration (causing maximal inhibition of auxin efflux) increased net auxin accumulation by ca. 194%, the maximum increase in net auxin accumulation in response to BFA, a vesicle trafficking inhibitor, was only around 35% (Petrášek et al., 2003; cf. Fig. 4). Therefore, there is a BFA-insensitive population of auxin efflux catalysts which remains sensitive to inhibition by NPA. In other words: in BY-2 tobacco cells, the inhibition of auxin efflux by NPA was more effective than that caused by BFA and was perhaps driven by an auxin-transport-specific mechanism.

The action of inhibitors of polar auxin transport on cytoskeletal structures and endoplasmic reticulum in tobacco cells

Observations on zucchini hypocotyls suggested an interaction between the cytoskeleton and components involved in auxin transport through a cell. Cox and Muday (1994) reported an association of NPA-binding activity with cytoskeleton and Butler et al. (1998) presented evidence that association of the NPA-binding protein with an intact actin cytoskeleton was necessary for maximal polar auxin transport *in vivo*. The latter suggestion was confirmed later by means of F-actin affinity chromatography (Hu et al., 2000). Actin-depolymerising drugs, such as cytochalasin D, interfered with polar auxin transport both in maize coleoptiles (Cande et al., 1973) and in *Arabidopsis* (Geldner et al., 2001). Thus the involvement of the cytoskeleton in general (Luschnig, 2001), and the actin filaments in particular (reviewed by Muday, 2000; Muday and DeLong, 2001; Muday and Murphy, 2002), in the auxin transport machinery is now well established.

The cycling of the putative auxin efflux carrier and its polar localisation seems to be an integral part of the mode of action of auxin (Friml et al., 2002b; reviews by Friml and Palme, 2002; Friml, 2003). Vesicle trafficking is involved in the site-directed traffic of auxin efflux carriers to the PM. The vesicle trafficking machinery functionally comprises the Golgi network, the ER and cytoskeletal structures, namely microfila-

ments in plants (Nebenfûhr et al., 2002). Taken together, it is obvious why BFA, an inhibitor of protein trafficking in the endomembrane system, interferes with polar auxin transport and increases the net accumulation of auxin in cells.

As already mentioned (see above), NPA recently has been reported to prevent BFA-induced internalisation of PIN1 protein, i.e. it interfered with PIN1 cycling between the PM and an endomembrane compartment (Geldner et al., 2001). NPA alone did not show any effect, even at very high concentration (150 μM), on PIN1 localisation in wild-type *Arabidopsis* roots (Gil et al., 2001). Interestingly, treatment of *tir3-1 Arabidopsis* mutant roots with NPA resulted in mislocalisation of PIN1 to an unidentified intracellular compartment, similar to the PIN1 internalisation induced by BFA. Mutants of the *tir3* type (**t**ransport **i**nhibitor **r**esponse **3**) display phenotypes consistent with decreased polar auxin transport and reduced NPA-binding activity. These results point to some kind of interaction between NPA, PIN1 localisation and vesicle trafficking. Since NPA (albeit at very high concentrations) also inhibited the internalisation of other proteins not directly connected with auxin transport (a PM-ATPase and KNOLLE, a syntaxin involved in vesicle docking), a non-specific and general interference with vesicle trafficking by auxin transport inhibitors has been suggested (Geldner et al., 2001; but see above).

One possibility was that NPA might interact with cell structures involved in vesicle-mediated traffic of proteins, such as the Golgi network, the ER or the cytoskeleton, perhaps in a manner similar to the action of BFA. However, treatment of BY-2 tobacco cells with NPA at concentrations at which its effect on auxin accumulation was maximal, had no effect on the arrangement of either the cytoskeleton or the ER (Petrášek et al., 2003). Even at concentrations one or two orders of magnitude higher than this, when the effect of NPA on auxin accumulation was already reversed, there were no observable changes in these intracellular structures. Parallel treatments with BFA, in contrast to NPA, revealed very significant effects of BFA on actin filaments and ER structure (Fig. 5) Therefore, if NPA interacts with vesicle trafficking, it must do so by a mechanism which is quite different from that of BFA. With respect to the concentration dependence of net auxin accumulation on NPA, we may speculate that NPA somehow interferes with a specific site-directed traffic of one or more components of the auxin efflux carrier complex.

The action of inhibitors of polar auxin transport on the growth of tobacco cells

In spite of the fact that the effects of NPA on polar auxin transport have been known for a long time, there is only very limited information about its effects on growth and development at the cell level. Since NPA inhibits auxin efflux from a cell and thus increases auxin concentration inside a cell, it presumably changes the internal hor-

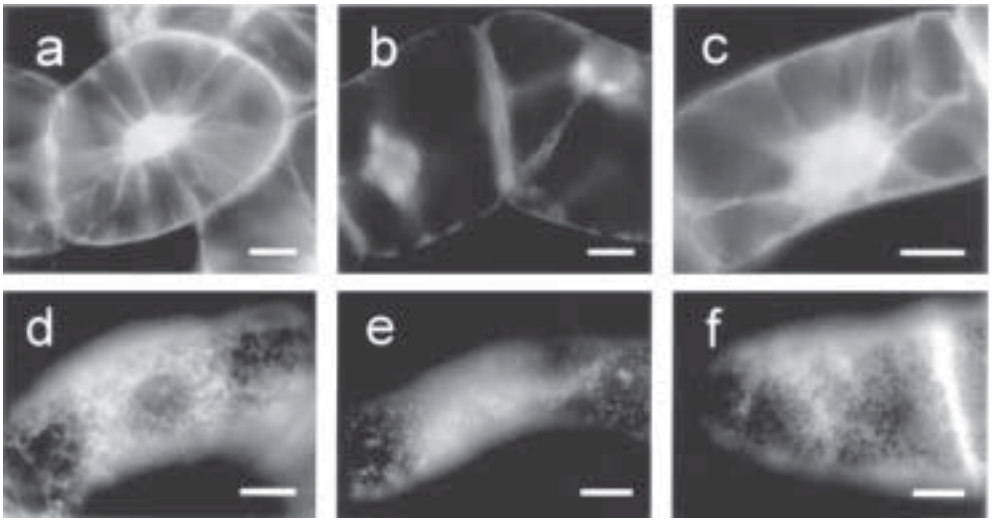

Fig. 5. The effect of brefeldin A (BFA) and 1-N-naphthylphthalamic acid (NPA) on the arrangement of actin filaments (AFs) and endoplasmic reticulum (ER) in 2-day-old cells of the BY-2 tobacco cell line. **a**, **b**, **c**. Rhodamin-phalloidin staining. **d**, **e**, **f**. Fluorescence of BY-2 cells expressing the ER-localised variant of green fluorescent protein (mGFP5-ER; Haseloff et al. 1997). mGFP5-ER contains ER-signalling and retention sequences. **a**,**d**. Control cells with radially oriented AFs in transvacuolar strands (**a**) and ER network in the cortical cytoplasm (**d**). **b**,**e**. AFs and cortical ER after 30 min incubation in 20 μM BFA. AFs staining pattern in perinuclear region (**b**), where AFs from transvacuolar strands are "pulled-down" to form clusters around the nucleus. Disintergration of the fine ER network in the cortical cytoplasm (**e**). **c**,**f.** AFs and cortical ER after 30 min incubation in 50 μM NPA. Unaltered pattern of staining of AFs in the perinuclear region (**c**). Unaltered ER in the cortical (**f**) cytoplasm. Scale bars = 10 μm. Modified from Petrášek et al. (2003).

monal balance quite significantly. The hormonal balance – especially the ratio auxins:cytokinins – is crucial for cell development (Skoog and Miller, 1957, and others, see above).

Auxin accumulation and auxin efflux carrier activity were studied during the growth cycle of the VBI-0 tobacco cell line (Petrášek et al., 2002b). Auxin accumulation underwent only relatively slight changes during a subculture period. However, the sensitivity of cells towards NPA changed markedly and reached a maximum when cells started to divide. Cultivation of cells in medium supplemented with NPA (10–50 μM, i.e. in the concentration range that had the maximum effect on the stimulation of auxin accumulation) resulted in a delay in the commencement of cell division. The delay was the same as if the cells were cultivated in the medium with auxin concentration ten times elevated. However, only when the cells were grown in the presence of NPA did they show changes in the orientation of cell division and abnormal polarity (Fig. 6). Thus it could be concluded that in tobacco cells the NPA-sensitive directed traffic of auxin efflux carriers to the PM might regulate the orientation of cell division (Petrášek et al., 2002b).

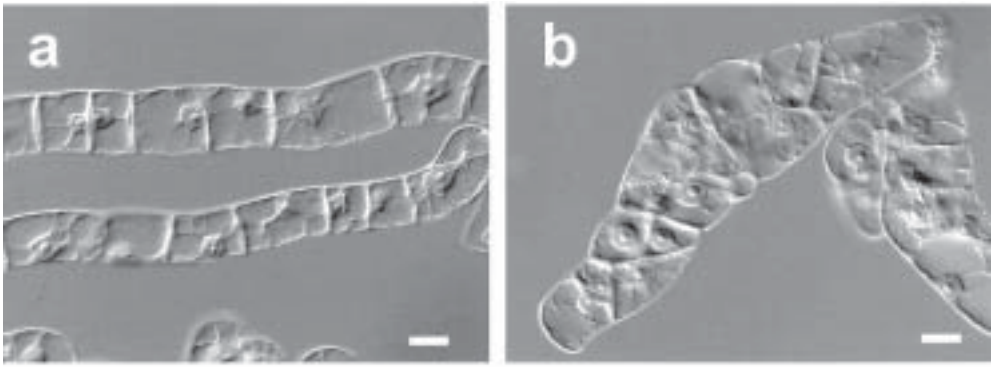

Fig. 6. Effect of 1-N-naphthylphthalamic acid (NPA) on the phenotype of suspension-cultured VBI-0 cells. **a**. Cells grown in control medium, day 9. **b**. Cells grown in control medium supplemented with NPA (final concentration 10 μM), day 9. Note abnormal cell division planes. Scale bars = 50 μm. Modified from Petrášek et al. (2002b).

Yasuhara and Shibaoka (2000) described the effects of BFA on suspension-cultured BY-2 tobacco cells. BFA treated cells could not complete cell division because of the inhibition of cell-plate formation due to the prevention of the depolymerisation of microtubules in the central region of the phragmoplast. Since treatment with BFA terminated cell development at the very beginning of the cell cycle, no effect on polarity of cell division could have been observed.

Conclusions

A summary of the effects of both NPA and BFA on auxin accumulation, on the arrangement of some intracellular structures and on polarity in suspension-cultured tobacco cells is given in Table 1. Both compounds increase net auxin accumulation - that is they inhibit efflux of auxin from the cells - but they seem to do so by different

Table 1. Summary of the effects of the polar auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA, 10–200 μM), and vesicle-trafficking inhibitor brefeldin A (BFA, 20–100 μM) on suspension-cultured tobacco cells. Compiled from Petrášek et al. (2002b, 2003) and Yasuhara & Shibaoka (2000).

	Auxin accumulation	Microtubules	Actin filaments	Endoplasmic reticulum	Polarity of cell division
NPA	+	-	-	-	+
BFA	+	-	+	+	n.a.

– No differences from control observed.
+ Auxin accumulation increased, structure affected, polarity changed.
n.a. Not applicable; cells cannot complete cell division (Yasuhara & Shibaoka 2000).

cellular mechanisms. When applied to the cultivation medium, NPA reversibly delays cell division. After recovery, the polarity of cell division is impaired; however, the cells are able to complete cell division and to survive. This is not the case for BFA, the application of which results in complete blockage of cell division. In contrast to BFA, NPA does not affect subcellular structures involved in vesicle trafficking in tobacco cells. However, it appears to interfere with the vesicle trafficking process in a way that has specific effects on auxin efflux. In agreement with Muday and DeLong (2001), Muday and Murphy (2002), and Luschnig (2001), the observations reviewed here strongly suggest that the NPA-binding protein (NBP) anchors the auxin efflux complex to actin filaments. Our own results suggest that NBP also directs this complex to the predetermined domain in the PM. Furthermore, NPA appears to interact with cellular structures that regulate the polarity of cell division.

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References

Baker, D.A., 2000a. Vascular transport of auxins and cytokinins in *Ricinus*. Plant Growth Regul., 32, 157–60.

Baker, D.A., 2000b. Long-distance vascular transport of endogenous hormones in plants and their role in source:sink regulation. Israel J. Plant Sci., 48, 199–203.

Benjamins, R., A. Quint, D. Weijers, P. Hooykaas, R. Offringa, 2001. The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. Development, 128, 4057–4067.

Bennett, M.J., A. Marchant, S. T. May, R. Swarup, 1998. Going the distance with auxin: unravelling the molecular basis of auxin transport. Philos. Trans. R. Soc. Lond. Ser. B, 353, 1511–1515.

Berleth, T. J. Mattsson, Ch. S. Hardtke, 2000. Vascular continuity, cell axialisation and auxin. Plant Growth Regul., 32, 173–185.

Butler, J. H., S. Q. Hu, S. R. Brady, M. W. Dixon, G. K. Muday, 1998. *In vitro* and *in vivo* evidence for actin association of the naphthylphthalamic acid-binding protein from zucchini hypocotyls. Plant J., 13, 291–301.

Cande, W. Z., M. H. M. Goldsmith, P. M. Ray, 1973. Polar auxin transport and auxin-induced elongation in the absence of cytoplasmic streaming. Planta, 111, 279–296.

Casimiro, I., A. Marchant, R. P. Bhalerao, T. Beeckman, S. Dhooge, R. Swarup, N. Graham, D. Inze, G. Sandberg, P. J. Casero, M. Bennett, 2001. Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell, 13, 843–852.

Chen, C. M., 1997. Cytokinin biosynthesis and interconversion. Physiol. Plant., 101, 665–673.

Chen, L., A. Ortiz-Lopez, A. Jung, D. R. Bush, 2001. *ANT1*, an aromatic and neutral amino acid transporter in Arabidopsis. Plant Physiol., 125, 1813–1820.

Cleland, R. E., 1995. Auxin and Cell Elongation. In Plant Hormones: Physiology, Biochemistry and Molecular Biology, Ed. P. J. Davies, Kluwer Academic Publishers, Dordrecht, 214–227.

Cox, D. N., G. K. Muday, 1994. NPA-binding activity is peripheral to the plasma-membrane and is associated with the cytoskeleton. Plant Cell, 6, 1941–1953.

D'Agostino, I. B., J. J. Kieber, 1999. Molecular mechanisms of cytokinin action. Curr. Opin. Plant Biol., 2, 359–364.

Davies, P. J., 1995. The plant hormone concept: Concentration, sensitivity and transport. In Plant Hormones: Physiology, Biochemistry and Molecular Biology, Ed. P. J. Davies, Kluwer Academic Publishers, Dordrecht, 13–38.

Delbarre, A., P. Muller, V. Imhoff, J. Guern, 1996. Comparison of the mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta, 198, 532–541.

Delbarre A, P. Muller, J. Guern, 1998. Short-lived and phosphorylated proteins contribute to carrier-mediated efflux, but not to influx, of auxin in suspension-cultured tobacco cells. Plant Physiol., 116, 833–844.

Depta, H., P. H. Rubery, 1984. A comparative study of carrier participation in the transport of 2,3,5-trriodobenzoic acid, indole-3-acetic acid, and 2,4-dichlorophenoxyacetic acid by *Cucurbita pepo* L. hypocotyl segments. J. Plant Physiol., 115, 371–387.

Depta, H., K.-H. Eisele, R. Hertel, 1983. Specific inhibitors of auxin transport: action on tissue segments and in vitro binding to membranes from maize coleoptiles. Plant Sci. Lett., 31, 181–192.

Deruère, J., K. Jackson, C. Garbers, D. Söll, A. DeLong, 1999. The RCN1-encoded A subunit of protein phosphatase 2A increases phosphatase activity in vivo. Plant J., 20, 389–399.

Friml, J., 2003. Auxin transport – shaping the plant. Curr. Opin. Plant. Biol., 6, 1–6.

Friml, J., K. Palme, 2002. Polar auxin transport - old questions and new concepts? Plant Mol. Biol., 49, 273–284.

Friml, J., E. Benková, I. Blilou, J. Wisniewska, T. Hamann, K. Ljung, S. Woody, G. Sandberg, B. Scheres, G. Jûrgens, K. Palme, 2002a. AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. Cell, 108, 661–673.

Friml, J., J. Wisniewska, E. Benková, K. Mendgen, K. Palme, 2002b. Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature, 415, 806–809.

Gälweiler, L., C. Guan, A. Müller, E. Wisman, K. Mendgen, A. Yephremov, K. Palme, 1998. Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science, 282, 2226–2230.

Garbers, C., A. DeLong, J. Deruère, P. Bernasconi, D. Söll, 1996. A mutation in protein phosphatase 2A regulatory subunit affects auxin transport in A*rabidopsis*. EMBO J., 15, 2115–2124.

Geldner, N., J. Friml, Y.-D. Stierhof, G. Jûrgens, K. Palme, 2001. Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature, 413, 425–428.

Gil, P., E. Dewey, J. Friml, Y. Zhao, K. C. Snowden, J. Putterill, K. Palme, M. Estelle, J. Chory, 2001. BIG: a callosin-like protein required for polar auxin transport in *Arabidopsis*. Genes Dev., 15, 1985–1997.

Goldsmith, M. H. M., 1977. The polar transport of auxin. Annu. Rev. Plant Physiol., 28, 439–478.

Haberer, G., J. J. Kieber, 2002. Cytokinins. New insights into a classic phytohormone. Plant Physiol., 128, 354–362.

Haseloff, J., K. R. Siemering, D. C. Prasher, S. Hodge, 1997. Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Sci. USA, 94, 2122–2127.

Hopkins, W. G., 1995. Introduction to Plant Physiology, John Wiley and Sons, New York, 316–331.

Hu, S. Q., S. R. Brady, D. R. Kovar, C. J. Staiger, G. B. Clark, S. J. Roux, G. K. Muday, 2000. Identification of plant actin binding proteins by F-actin affinity chromatography. Plant J., 24, 127–137.

Imhoff, V., P. Muller, J. Guern, A. Delbarre, 2000. Inhibitors of the carrier-mediated influx of auxin in suspension-cultured tobacco cells. Planta, 210, 580–588.

Kamínek, M., 1992. Progress in cytokinin research. Trends Biotechnol., 10, 159–164.

Katekar, G. F., A. E. Geissler, 1980. Auxin transport inhibitors: IV. Evidence of a common mode of action for a proposed class of auxin transport inhibitors: The phytotropins. Plant Physiol., 66, 1190–1195.

Kumagai, F., S. Hasezawa, 2001. Dynamic organization of microtubules and microfilaments during cell cycle progression in higher plant cells. Plant Biol., 3, 4–16.

Lomax, T. L., G. K. Muday, P. H. Rubery, 1995. Auxin transport. In: Plant Hormones: Physiology, Biochemistry and Molecular Biology, Ed. P. J. Davies, Kluwer Academic Publishers, Dordrecht, 509–530.

Luschnig, C., 2001. Auxin transport: why plants like to think BIG. Curr. Biol., 11, 831–833.

Luschnig, C., 2002. Auxin transport: ABC proteins join the club. Trends Plant Sci., 7, 329–332.

Morris, D. A., 2000. Transmembrane auxin carrier systems – dynamic regulators of polar auxin transport. Plant Growth Regul., 32, 161–172.

Morris, D. A., J. S. Robinson, 1998. Targeting of auxin carriers to the plasma membrane: differential effects of brefeldin A on the traffic of auxin uptake and efflux carriers. Planta, 205, 606–612.

Morris, D. A., P. H. Rubery, J. Jarman, M. Sabater, 1991. Effects of inhibitors of protein synthesis on transmembrane auxin transport in *Cucurbita pepo* L. hypocotyl segments. J. Exp. Bot., 42, 773–783.

Muday, G. K., 2000. Maintenance of asymmetric cellular localization of an auxin transport protein through interaction with the actin cytoskeleton. J. Plant Growth Regul., 19, 385–396.

Muday, G.K., 2001. Auxins and tropisms. J. Plant Growth Regul., 20, 226–243..

Muday, G. K., A. DeLong, 2001. Polar auxin transport: controlling where and how much. Trends Plant Sci., 6, 535–542.

Muday, G. K., A.S. Murphy, 2002. An emerging model of auxin transport regulation. Plant Cell, 14, 293–299.

Nagata, T., Y. Nemoto, S. Hasezawa, 1992. Tobacco BY-2 cell line as the "Hela" cell in the cell biology of higher plants. Int. Rev. Cytol., 132, 1–30.

Nebenführ, A., C. Ritzenthaler, D. G. Robinson, 2002. Brefeldin A: Deciphering an enigmatic inhibitor of secretion. Plant Physiol., 130, 1102–1108.

Noh, B., A. S. Murphy, E. P. Spalding, 2001. *Multidrug resistance*-like genes of *Arabidopsis* required for auxin transport and auxin-mediated development. Plant Cell, 13, 2441–2454.

Opatrný, Z., J. Opatrná, 1976. The specificity of the effect of 2,4-D and NAA on the growth, micromorphology, and the occurrence of starch in long-term *Nicotiana tabacum* cell strains. Biol. Plant., 18, 381–400.

Parry, G., A. Delbarre, A. Marchant, R. Swarup, R. Napier, C. Perrot-Rechenmann, M. Bennett, 2001. Novel auxin transport inhibitors phenocopy the auxin influx carrier mutation *aux1*. Plant J., 25, 399–406.

Pasternak, T., P. Miskolczi, F. Ayaydin, T. Mészáros, D. Dudits, A. Fehér, 2000. Exogenous auxin and cytokinin dependent activation of CDKs and cell division in leaf protoplast-derived cells of alfalfa. Plant Growth Regul., 32, 129–141.

Petrášek, J., A. Freudenreich, A. Heuing, Z. Opatrný, P. Nick, 1998. Heat-shock protein 90 is associated with microtubules in tobacco cells. Protoplasma, 202, 161–174.

Petrášek, J., A. Březinová, J. Holík, E. Zažímalová, 2002a. Excretion of cytokinins to the cultivation medium by the suspension-cultured tobacco cells. Plant Cell Rep., 21, 97–104.

Petrášek, J., M. Elèkner, D. A. Morris, E. Zažímalová, 2002b. Auxin efflux carrier activity and auxin accumulation regulate cell division and polarity in tobacco cells. Planta, 216, 302–308.

Petrášek, J., A. Černá, K. Schwarzerová, M. Elèkner, D. A. Morris, E. Zažímalová, 2003. Do phytotropins inhibit auxin efflux by impairing vesicle traffic? Plant Physiol., 131, 254–263.

Raven, J. A. Transport of indoleacetic acid in plant cells in relation to pH and electrical potential gradients, and its significance for polar IAA transport. New Phytol., 74, 163–174.

Robinson, J. S., A. C. Albert, D. A. Morris, 1999. Differential effects of brefeldin A and cycloheximide on the activity of auxin efflux carriers in *Cucurbita pepo* L. J. Plant Physiol., 155, 678–684.

Rubery, P. H., 1990. Phytotropins: receptors and endogenous ligands. Symp. Soc. Exp. Biol., 44, 119–146.

Rubery, P. H., A. R. Sheldrake, 1974. Carrier-mediated auxin transport. Planta, 188, 101–121.

Schwarzerová, K., S. Zelenková, P. Nick, Z. Opatrny, 2002. Aluminum-induced rapid changes in the microtubular cytoskeleton of tobacco cell lines. Plant Cell Physiol., 43, 207–216.

Skoog, F., C. Miller, 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symposia of the Society for Experimental Biology, No. XI, The Biological Action of Growth Substances, 118–140.

Went, F. W., 1928. Wuchstoff und Wachstum. Rec. Trav. Bot. Néer., 25, 1–116.

Wilkinson, S., D. A. Morris, 1994. Targeting of auxin carriers to the plasma membrane: effects of monensin on transmembrane auxin transport in *Cucurbita pepo* L. tissue. Planta, 193, 194–202.

Yasuhara, H., H. Shibaoka, 2000. Inhibition of cell-plate formation by brefeldin A inhibited the depolymerization of microtubules in the central region of the phragmoplast. Plant Cell Physiol., 41, 300–310.

Zažímalová, E., Z. Opatrný, A. Březinová, J. Eder, 1995. The effect of auxin starvation on the growth of auxin-dependent tobacco cell culture: dynamics of auxin reception and endogenous free IAA content. J. Exp. Bot., 46, 1205–1213.