E. THE FUNCTIONING OF HORMONES IN PLANT GROWTH AND DEVELOPMENT

E1. The Transport of Auxins

David A. Morris[1], Jiří Friml[2], and Eva Zažímalová[3]

[1]School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton, SO16 7PX, UK. [2]Centre for Plant Molecular Biology, University of Tübingen, Auf der Morgenstelle 3, 72076 Tübingen, Germany. [3]Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 135, 16502 Prague 6, Czech Republic.

E-mail: dam3@soton.ac.uk

INTRODUCTION

Auxins play a crucial role in the regulation of spatial and temporal aspects of plant growth and development[1]. As well as being required for the division, enlargement and differentiation of individual plant cells, auxins also function as signals between cells, tissues and organs. In this way they contribute to the coordination and integration of growth and development in the whole plant and to physiological responses of plants to environmental cues (63). At the individual cell level, fast changes or pulses in hormone concentration may function to initiate or to terminate a developmental process. In contrast, the maintenance of a stable concentration of the hormone (homeostasis) may be necessary to maintain the progress of a developmental event that has already been initiated. It should be stressed that both transmembrane transport and metabolic processes such as biosynthesis, degradation and conjugation,

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[1] Abbreviations: ARF, auxin response factor; ABC, ATP-binding cassette; BFA, brefeldin A; CHX, cycloheximide;   2,4-D, 2,4-dichlorophenoxyacetic acid;   GEF, guanine nucleotide exchange factor; MNK, Menkes copper-transporting ATPase; NAA, 1-naphthaleneactic acid; NPA, 1-N-naphthylphthalamic acid; NBP, NPA-binding protein; PM, plasma membrane; TIBA, 2,3,5-triiodobenzoic acid.

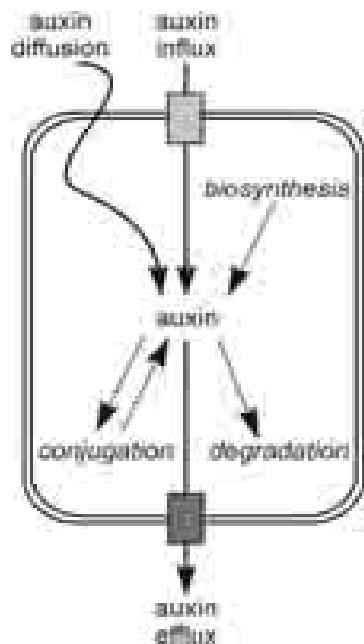operate together to regulate the momentary concentration of the active hormone molecule in target cells and cell compartments (97; Fig. 1).

Auxins such as indole-3-acetic acid (IAA) mediate interactions between cells, tissues and organs over both short distances (for example, between adjacent cells) and over very long ones (for example between the shoot apex and sites of lateral root initiation). Characteristically, auxin transport through cells and tissues other than mature vascular elements is strongly polar. This feature endows an auxin signal with directional properties and, *inter alia*, the vectorial transport of IAA is important in the regulation of spatial aspects of plant development. Consequently IAA transport may play a crucial role in the initiation and/or maintenance of cell and tissue polarity and axiality, upon which pattern formation depends (31).

New approaches to the physiology of auxin transport, and rapid advances in our knowledge of the molecular and genetic mechanisms that underlie it, have dramatically increased our understanding of the process in the nine years that have elapsed since auxin transport was discussed in the last edition of this book (48). It has become increasingly clear that polar auxin transport is a very dynamic and complex process and one that is regulated at many different levels.

Figure 1. Scheme of the metabolic and transport processes involved in regulating the momentary level of auxin in a plant cell or its compartments. Full arrows: transport processes; dotted arrows: metabolic processes.

This chapter describes and analyses these recent developments. The earlier work on which they were based has been reviewed elsewhere (33, 42); several comprehensive reviews analyse the recent discoveries relating to the mechanism, regulation, molecular genetics and significance in development of polar auxin transport (4, 19, 22, 59, 63, 64, 97). The reader is referred to these for further background information.

It is impossible to discuss polar auxin transport without reference to the direction of transport in the plant and the orientation of auxin-transporting cells with respect to plant poles and axes. The terminology in general use has the potential to confuse, not least because in plant developmental biology the base of the developing embryo equates to the apex (i.e. the youngest, meristematic, end) of the growing root. Thus the basal end of a cell refers to
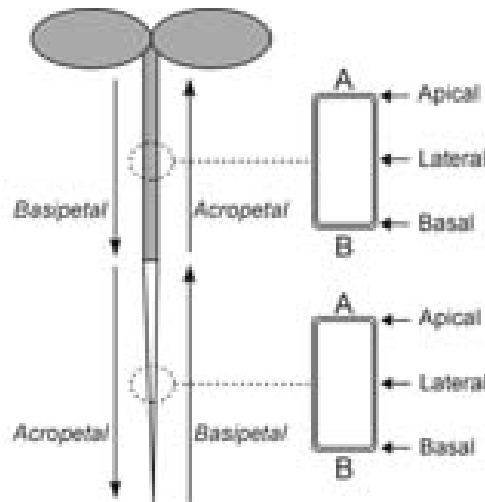
Figure 2. The nomenclature used in the text to define the directions of auxin transport in a plant or plant organ and apical and basal ends of root and shoot cells. The apical end of a cell is defined here as the end nearest the shoot tip, regardless of whether the cell is located in the root or the shoot. **A** = Apical end of cell; **B** = basal end of cell.

the end nearest the root pole and the apical end to that nearest the shoot pole, *regardless of whether the cell is in the root or shoot*. In roots, this contrasts with the terms acropetal (towards the root tip) and basipetal (towards the base of the root), generally used to denote direction. To avoid possible confusion, the terminology that has been adopted throughout this chapter is defined in Fig. 2.

LONG-DISTANCE AUXIN MOVEMENT - PATHWAYS AND MECHANISMS

Two physiologically distinct and spatially separated pathways function to transport auxin over long distances through plants. Firstly, auxin is translocated rapidly by mass flow with other metabolites in the mature phloem. Secondly, auxin is transported downwards towards the root tips from immature tissues close to the shoot apex by a much slower (ca. 7-15 mm h^{-1}), carrier-dependent, cell-to-cell polar transport (33). Early experiments (14) suggested that some of the auxin reaching the root tip might be re-exported basipetally through the root, possibly in the cortex.

Non-Polar Translocation of Auxin in Vascular Tissues

IAA is a natural constituent of phloem sap and is exported in physiologically significant quantities by source leaves (1). Furthermore, labeled auxins applied directly to source leaves, and IAA synthesized by source leaves from applied tryptophan, are also rapidly loaded into the phloem and exported (1, and references therein). Only traces of endogenous IAA are normally found in xylem sap (1) and it is unlikely that the mature xylem functions as a major pathway for long-distance auxin movement.

The IAA that is loaded into mature phloem in source leaves is translocated passively in solution in the phloem sap to sink organs and tissues where unloading of phloem-mobile assimilates occurs. Thus the direction and speed of auxin translocation in this pathway will be subject to all the

factors that influence phloem translocation (58). This makes the phloem an unlikely route for the transmission of a signal molecule involved in the fine-tuning of growth and development. Nevertheless, significant quantities of IAA occur in phloem sap and it is clear that the total amount of auxin that is delivered by the phloem to sink tissues is considerable. Consequently, the high concentration of IAA detected in young (sink) leaves at the shoot apex (often uncritically assumed to indicate high rates of local biosynthesis of IAA) results from both local synthesis (47) and IAA accumulation following unloading of phloem-mobile auxin synthesized in source leaves (1).

In addition to having roles in the loading and unloading of phloem mobile assimilates in source and sink tissues (58), the IAA translocated in the phloem also is a major source of the IAA transported in the long distance polar auxin transport pathway (see below).

The Long Distance Polar Auxin Transport Pathway

Auxin exported by the apical tissues of intact dicotyledonous plants moves downwards through the stem and root by a mechanism that has all the characteristics of the slow, polar transport first described from coleoptile and stem segments (33, 59). In stems, this transport occurs basipetally, but not acropetally (Fig. 2), at velocities around 10 mm h^{-1}; is blocked by inhibitors of polar auxin transport (see below); and is competed by other growth-active auxins such as 1-naphthaleneactic (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). In both herbaceous and woody dicotyledonous species the pathway for the root-directed long distance polar transport of auxin includes the vascular cambium and its partially differentiated derivatives, including differentiating xylem vessels and xylem parenchyma (44, 62). Environmental treatments and experimental conditions that reduce or inhibit cambial activity correspondingly reduce long distance polar auxin transport.

Communication Between Polar and Non-Polar Pathways

Of particular interest and importance is the question of whether the two long-distance auxin transport pathways – the polar pathway in immature cells and the translocation pathway in mature vascular elements – are isolated from each other, or whether auxin moving in one pathway can enter the other. The answer to this question has considerable bearing on the sources of auxin present in the polar transport pathway.

There is little evidence to indicate that auxin enters the phloem from the polar transport pathway; on the contrary, a number of reports suggest that it does not (10). In contrast, there is both physiological and genetic evidence that phloem-translocated auxin is transferred to the long distance polar auxin transport system (10). For example, when [1-^{14}C]IAA was applied to a mature leaf of pea (*Pisum sativum* L.) it was exported in the phloem (indicated by recovery of label in aphid colonies on the internodes above and below the fed leaf). However, efflux of [^{14}C] from 30 mm stem segments

excised 4h after labelling occurred in the basipetal, but not in the acropetal, direction and was prevented by inclusion of 1-N-naphthylphthalamic acid (NPA), an inhibitor of polar auxin transport (see later). This polar, NPA-sensitive efflux clearly suggests that auxin had entered the polar transport system from the phloem. The quantity of auxin that effluxed basipetally from the stem segments was dramatically reduced when very young tissues at the shoot apex were excised, suggesting that the transfer of auxin from the phloem to the polar transport pathway occurred mainly in the younger sink tissues of the shoot apex. A possible explanation for this is that the transfer of IAA from the phloem to the polar transport system may require carrier-mediated radial transfer through undifferentiated cells.

PHYSIOLOGICAL ASPECTS OF POLAR TRANSPORT

Carrier-Mediated Auxin Transport: The Role of Efflux Carriers

An early indication that polar auxin movement involved carrier-mediated cell-to-cell transport was the observation that 2,3,5-triiodobenzoic acid (TIBA), a known inhibitor of polar auxin transport, stimulated the net uptake of labelled IAA in *Zea mays* L. coleoptile segments (37). This indicated that efflux rather than influx of IAA was inhibited by TIBA and therefore that efflux was more important than uptake in determining polar transport. Together with the already known facts that polar transport could take place against an overall concentration gradient, was sensitive to inhibitors of energy metabolism, and could continue in the absence of cytoplasmic continuity between adjacent cells (33, 42), these observations led to suggestions that auxin uptake and efflux by cells were mediated by energy-dependent influx and efflux carriers (37). Polarity of transport was believed to result from small differences in net auxin efflux between the two ends of a cell, and polar transport was envisaged as involving active (polar) "secretion" of auxin by carriers from the end of one cell, diffusion of auxin through the intervening wall space, and active uptake of auxin by an adjacent cell.

An important milestone in the efforts to explain the mechanism of polar auxin transport was the proposal that net polar transport of auxin through cells could be explained by differences between their two ends in their relative permeabilities to dissociated and undissociated auxin molecules (76, 79; Fig. 3). Being relatively lipophilic, undissociated molecules of some auxin species (e.g. IAA and NAA, but not 2-4,D) readily enter cells by diffusion across the plasma membrane (PM) from the surrounding wall space. Because the cytoplasm is normally far less acid than the wall space (typically around pH 7.0 and pH 5.5 respectively), a high proportion of the auxin molecules which enter the cells by diffusion dissociate after crossing the PM, the extent to which they do so depending on the dissociation constant of the auxin species concerned. Because auxin anions cannot readily penetrate the PM, they become "trapped" in the cytoplasm. Undissociated auxin will

continue to move into the cell as long as an inwardly directed concentration gradient in undissociated auxin persists. As concentration equilibrium is approached, the total concentration of auxin in the cell (dissociated plus undissociated) will exceed the total concentration in the more acidic wall space, where the concentration of auxin anions will be considerably lower. Thus, when a pH gradient is maintained across the PM, diffusion alone can appear to drive accumulation of auxin against a gradient in its concentration! Unlike the polar secretion model (see above), which assumes primary activation of the transport catalysts, the "chemiosmotic polar diffusion model" requires only secondary energy expenditure in order to maintain the transmembrane pH gradient (Fig. 3).

As in the polar secretion model, an asymmetric distribution of auxin anion efflux carriers in the PM will lead to a polarized leakage of auxin anions from a cell (79), and if the asymmetry of carrier distribution is repeated in each cell in a file of contiguous cells, it would amplify the polarized leakage of auxin and lead to a net polar movement of auxin through tissue.

Figure 3. Components of transmembrane auxin transport according to the chemiosmotic polar diffusion model (33). A membrane pH gradient (maintained by plasma membrane H^+-ATPases) drives diffusive accumulation of undissociated auxin molecules. At the higher pH of the cytoplasm, some of the auxin molecules which enter the cell dissociate. The plasma membrane is relatively impermeable to auxin anions (IAA^-), which are "trapped" in the cytoplasm and can only exit or enter the cell through the action of specific influx (upper; light shading) and efflux (lower; heavy shading) carrier systems. Asymmetry in the distribution of the two carrier systems, more especially the efflux carrier, results in a net polar transport of auxin through the cell.

Auxin Uptake Carriers

Despite the prominence given to the efflux carrier in polar transport, it quickly became clear that a functionally distinct class of auxin anion carriers is also involved in auxin uptake. This was first suggested by observations that auxin uptake by suspension cultured cells (79) and tissue segments (15), possessed a saturable component. Elegant experiments demonstrated that auxin uptake into sealed outside-out zucchini (*Cucurbita pepo* L.) hypocotyl PM vesicles was a saturable, carrier-mediated process specific for growth
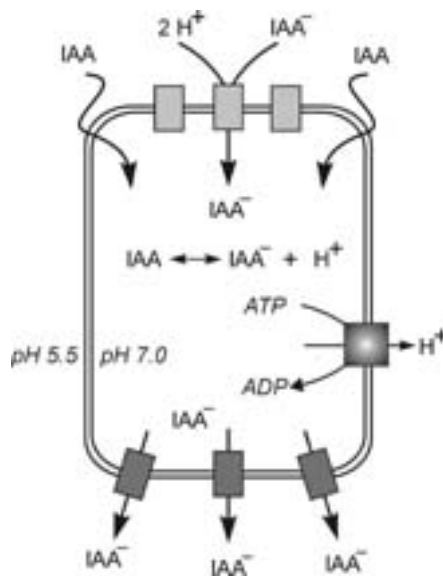
active auxins. Subsequently, it was found that carrier-mediated uptake was electrogenic and probably involved a proton symport in which two protons were transported for each IAA⁻ anion (48).

Although undissociated IAA can readily enter cells by diffusion across the PM, at the low concentrations of endogenous IAA likely to be present in the extracellular wall space in intact plants, it is probable that high affinity uptake carriers provide a more efficient means of effecting auxin uptake than diffusion. A key role for auxin influx carriers in polar auxin transport and in the loading of auxin into and unloading from the long distance transport pathway in the phloem is now supported both by physiological and biochemical observations and by evidence from recent molecular genetics studies (discussed in detail later).

Auxin Efflux Carriers Are Multi-Component Systems

It is probable that auxin efflux carriers are multi-component systems consisting of at least two, but possibly more individual protein components, each with a different function (61). The synthetic auxin transport inhibitor NPA strongly inhibits auxin efflux and consequently stimulates auxin accumulation by cells (59, 78). The mechanism by which NPA inhibits polar auxin transport remains a matter of debate and will be discussed later in this chapter, but available evidence suggests that it is mediated by a specific, high affinity, NPA-binding protein (NBP; 78). Protein synthesis inhibitors such as cycloheximide (CHX) rapidly uncouple carrier-mediated auxin efflux from the inhibition of efflux by NPA, but in the short-term do not affect auxin efflux itself or the saturable binding of NPA to microsomal membranes (61). These observations suggest that the NBP and the efflux catalyst are separate proteins that may interact through a third, rapidly turned over transducing protein (16, 49, 59, 63).
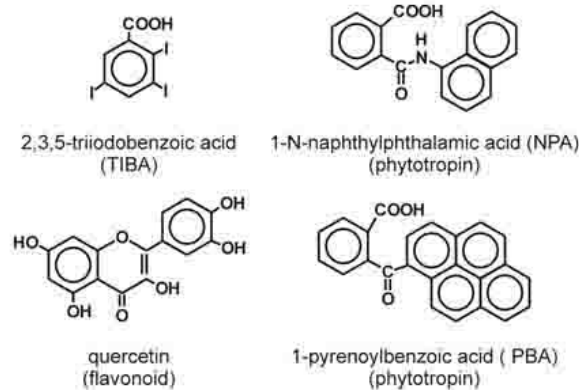
Polar Auxin Transport Inhibitors and NPA-Binding Proteins

Inhibitors of polar auxin transport (Fig. 4) have been very useful tools with which to investigate both the mechanism and regulation of polar transport and the role auxin transport plays in the control of plant

Figure 4. Examples of inhibitors of polar auxin transport. Of these, only quercetin occurs naturally.

development. The auxin transport inhibitors NPA and TIBA have been mentioned already. NPA, the most widely used of these, belongs to a class of structurally related synthetic compounds – phytotropins – characterized by their inhibition of auxin-dependent tropic responses, (78). Most reports indicate that the phytotropins interfere with polar auxin transport by strongly inhibiting auxin efflux from cells and a characteristic feature of their action is that auxin accumulates in treated cells. When stems of intact plants are ringed with a preparation containing NPA, root-directed basipetal transport of auxin is blocked and auxin accumulates in tissues above the NPA-treated region (e.g. 10). Despite the fact that most responses to NPA are best interpreted in terms of inhibition of efflux, a few reports suggest that under some circumstances influx carrier activity may also be sensitive to NPA (17).

Phytotropin action is mediated through a putative phytotropin-binding (NPA-binding) receptor protein, the NBP. For reasons already discussed, it is likely that the NBP is a separately synthesised and targeted regulatory protein that is functionally associated with the efflux catalyst. Interestingly, an NBP detected by an indirect immunofluorescence technique, using monoclonal antibodies raised against a membrane associated NPA-binding protein from zucchini hypocotyl tissue, exhibited a predominantly polar (basal) localization in cells associated with the vascular tissue in pea stem sections (40). This was the first direct evidence for the polar localization in cells of a protein believed to be associated with polar auxin transport.

Despite one report that the NBP is an integral membrane protein (8), the weight of evidence indicates that the NBP is probably a peripheral membrane protein that is located on the cytoplasmic face of the PM and is associated with the actin cytoskeleton (63, 64). Several studies have demonstrated that TIBA does not compete with NPA for high affinity binding sites on the NBP, implying the probable existence of different binding sites for different classes of polar auxin transport inhibitors.

All the first-discovered polar auxin transport inhibitors were synthetic compounds. Nevertheless, NBPs are almost universally distributed in the plant kingdom, suggesting that the NBP performs an essential function in plant cells. Although this function may not necessarily involve the regulation of polar auxin transport, the possibility remains that natural equivalents of the phytotropins exist and that through association with the NPA-binding site, they regulate auxin carriers. A screen for phenolic compounds which both promoted net accumulation of labelled IAA by zucchini hypocotyls segments and competed with labelled NPA for binding sites on membrane fractions isolated from zucchini hypocotyls, led to the discovery of a group of naturally-occurring flavonoids which were both ligands for the NPA binding site and inhibitors of IAA efflux (41). When several of these compounds were compared, their ability to inhibit NPA binding was directly correlated with their ability to stimulate IAA accumulation (41). Flavonoids, therefore, might be natural regulators of auxin efflux carriers. In support of this, the flavonoid-deficient Arabidopsis mutant *tt4* (*transparent testa 4*) was found to

have altered patterns of auxin distribution resulting from higher than normal auxin efflux (67). The lesion in auxin transport could be corrected by application of the missing intermediate in the flavonoid biosynthetic pathway, naringenin.

Rapid Turnover and Cycling of the Auxin Efflux Carrier

A significant recent discovery was that auxin efflux catalysts turnover very rapidly in the PM. This was first revealed by investigations of the effect of the inhibitors of vesicle traffic to the PM, monensin and brefeldin A (BFA), on the accumulation of IAA by zucchini (*Cucurbita pepo* L.) hypocotyl segments (59, 77; Fig. 5), and of the effect of BFA on NAA accumulation by suspension-cultured tobacco cells (*Nicotiana tabacum* L.; 16). Both monensin and BFA very rapidly stimulated IAA or NAA accumulation (depending on system), but in neither system did they affect the accumulation of 2,4-D (16, 60). Because 2,4-D is a readily transported substrate for auxin uptake carriers but not, in most species, for auxin efflux carriers (17), these observations indicated that the target for BFA action was the efflux carrier system. This is supported by an observed reduction in the efflux of NAA or IAA (but not of 2,4-D) from pre-loaded cells and tissue segments in the presence of BFA (16, 60). Also consistent with this conclusion is the ability of BFA to abolish p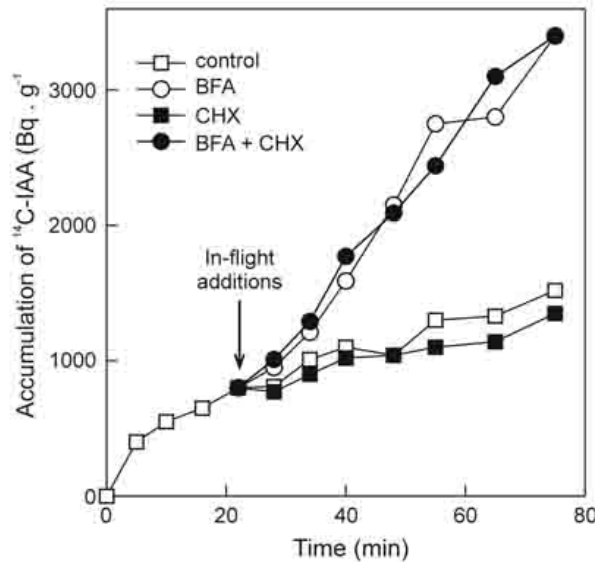olar (basipetal) auxin transport in long (30 mm) pea and zucchini stem segments (77). Significantly, BFA does not affect saturable NPA binding to microsomal preparations from zucchini providing additional evidence that the NBP and the auxin efflux catalyst are different proteins (77). Compared with responses to CHX, the lag time for which may be up to 2.0 h in zucchini, the lag for BFA action is very short (minutes or less; 16, 77). Furthermore, the

Figure 5. The effect of inflight additions (arrow; 22 min from start) of brefeldin A (BFA; 30 μM)) and cycloheximide (CHX; 10 μM) on the time-course of accumulation of [^{14}C]IAA by 2 mm zucchini hypocotyl segments. (Redrawn from 77).

response to BFA is unaffected by CHX (Fig. 5).

Taken together, these observations provided strong indirect evidence that an essential component of the auxin efflux carrier system (possibly the efflux catalyst itself) is targeted to the PM through the BFA-sensitive secretory system, and that this component turns over very rapidly at the PM without the need for concurrent protein synthesis; this implies the existence of internal pools of the carrier protein (16, 60, 77). It has been argued that because such pools would be of finite capacity, the high rates of efflux carrier turnover revealed by these experiments could only be sustained if a proportion of the carriers cycled between the PM and the proposed pools (77).

Rapid cycling of PM-located carriers and receptors in animal cell systems is well documented. Examples of particular interest in relation to the auxin efflux carriers include the Menkes copper-transporting ATPase (MNK) and the insulin-dependent GLUT4 glucose transporter. Similarities exist between the cycling of GLUT4 in insulin-sensitive mammalian cells and the putative cycling of auxin efflux catalysts in plant cells (59, 64). Cycling of GLUT4 involves an endosomal pathway, whilst MNK cycles between the PM and the *trans* Golgi network. The steady-state distribution of MNK protein between the PM and the *trans* Golgi network depends on substrate (Cu ions) levels in the cell (59). This property is of particular interest because there is indirect evidence to show that the targeting of auxin carriers to the PM also may be stimulated by the carrier substrate itself, namely IAA (59).

The Role of the Cytoskeleton in Auxin Carrier Traffic and Cycling

The actin cytoskeleton plays an important role in directing vesicular traffic to delivery sites at the PM and to other membrane-enclosed compartments in the cell. It has been known for several years that the NBP is closely associated with actin filaments (49, 63, 64).

The link between the high affinity NBP and the actin cytoskeleton suggests that NBPs may play a role in directing the polar distribution of auxin efflux carriers, which have been shown to cycle along actin filaments (30). However, the link remains to be proven experimentally.

Irrespective of whether NPA acts through the NBP, NPA action does not involve changes in the structure of the actin cytoskeleton; NPA has been shown recently to have no effect on the arrangement of either microtubules or actin filaments in BY-2 tobacco cells, even at concentrations well above that required to saturate the inhibition of auxin efflux (72).

THE GENETICS OF AUXIN TRANSPORT

Although physiological and biochemical approaches have provided much information about the process of polar auxin transport and its role in plant development, they have told us little about the molecular identity and

molecular regulation of components of the polar auxin transport system. Recent developments in plant molecular genetics, particularly the analysis of Arabidopsis mutants affected in polar transport, or in responses to auxins or auxin transport inhibitors, have enabled major advances to be made in our understanding of the process. Some of these developments are now described.

AUX1 Proteins – Components of Auxin Influx

A mutant referred to as *auxin 1* (*aux1*) has been pivotal in attempts to identify genes that encode auxin influx carrier catalysts (3). The root agravitropic and auxin-resistant phenotype of *aux1* (Fig. 6B) is consistent with a defect in auxin influx, but similar phenotypes have been observed also in mutants defective in auxin response (46). The *AUX1* gene encodes a protein that shares significant similarity with plant amino acid permeases – this favours a role for AUX1 in the uptake of the tryptophan-like IAA (3). Despite the fact that final biochemical proof of AUX1 function as an auxin uptake carrier is still lacking, several lines of evidence strongly support its involvement in auxin influx. The strongest support comes from a detailed analysis of the *aux1* phenotype. It has been demonstrated that the membrane permeable NAA rescues the *aux1* root agravitropic phenotype much more efficiently than the less membrane permeable IAA or 2,4-D and that this rescue coincides with restoration of polar auxin transport in this mutant (53, 96). Moreover, the *aux1* phenotype can be mimicked by growing seedlings on media containing the recently identified (39) inhibitors of auxin influx, 1-naphthoxyacetic and 3-chloro-4-hydroxyphenylacetic acids (71). The most direct support for AUX1 as an influx component comes from auxin uptake assays in *aux1* and wild type roots. Roots of *aux1* accumulated significantly less auxin influx substrate 2,4-D than do wild-type roots but no such difference was found for the membrane-permeable NAA or the IAA-like tryptophan (53).

Recently the AUX1 protein was localized within Arabidopsis root tissue using an epitope tagging approach (86). It was detected in a remarkable pattern in a subset of protophloem, columella, lateral root cap and epidermal cells, exclusively in the root tips. Most interestingly, the polar localization of AUX1 has been detected in the protophloem cells at the apical side opposite to that of PIN1 protein (Fig. 6C). However, the root tips of *aux1* contain less free auxin than wild type, probably due to defects in long distance auxin distribution from apex to the root. This paradox between local expression and long-distance effect of AUX1, taken together with its localization at the upper side of protophloem cells, suggests a role of AUX1 protein in the protophloem in unloading auxin from the mature phloem into the polar auxin transport system in the root tip. *AUX1* is a member of the small gene family in Arabidopsis. However the characterization of the other members of this family has not yet been reported (87).
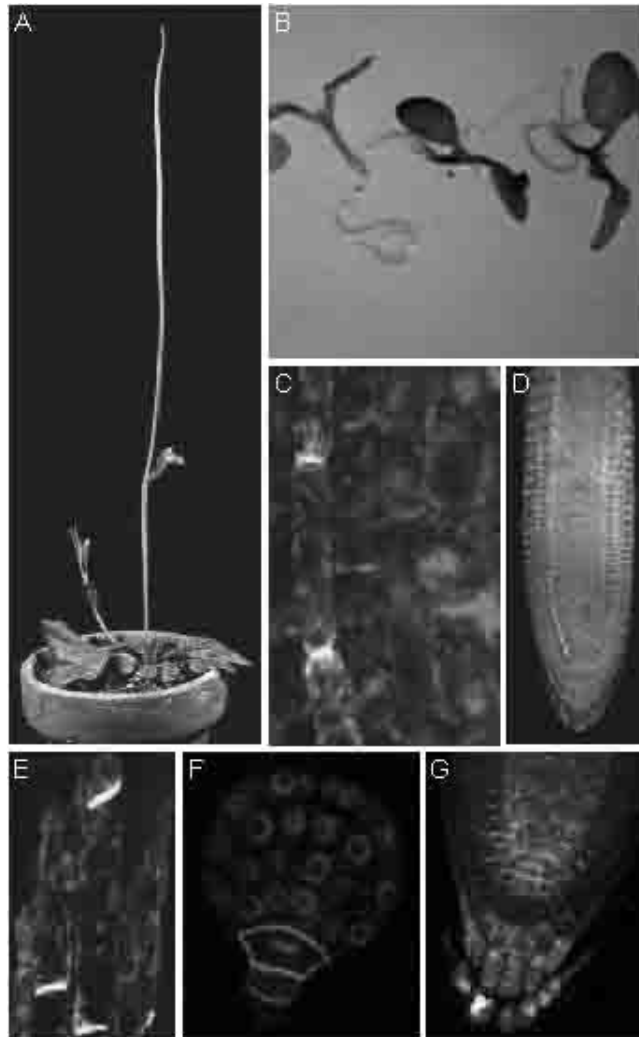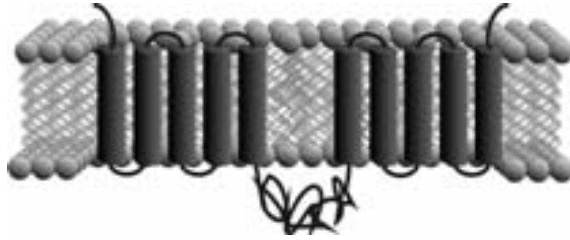
Figure 6 (Color plate on page xxx). A. The "knitting needle" like phenotype of the Arabidopsis *pin* mutant. B. Root agravitropic phenotype of the Arabidopsis *aux1* mutant. C. Polar localization of the putative auxin influx carrier AUX1 (green) at the apical side and the putative auxin efflux carrier PIN1 (red) at the basal side of protophloem cells in an Arabidopsis root. D. Polar localization of PIN2 (green) at the apical side of lateral root cap and epidermis cells in an Arabidopsis root. The less polar localization of PIN2 in cells of the cortex is also visible. E. Basal localization of PIN1 (yellow) in xylem parenchyma cells in a longitudinal section of an Arabidopsis inflorescence. F. PIN4 localization (red) around the hypophysis and at the basal side of the subtending suspensor cell, in a globular stage Arabidopsis embryo. DAPI stained nuclei are depicted in blue. G. PIN4 localization (green) in the central root meristem. Basal localization in vascular and endodermis/cortex initials and their daughter cells; less pronounced basal localization in the quiescent centre; and non-polar localization in columella cells. (A, E from 70; B, C from 86; D from 21; F, G from 20).

Figure 7. Predicted topology of the AtPIN1 protein showing conserved transmembrane domains at the N- and C-termini and the large, central, hydrophilic loop.

PIN Proteins – Components of the Auxin Efflux Machinery

In the early nineties, the knitting needle-like *pin-formed 1* (*pin1*) mutant phenotype of Arabidopsis was described (Fig. 6A). This strongly resembled plants treated with inhibitors of auxin efflux. Furthermore, basipetal auxin transport in this mutant is strongly reduced (69). The *AtPIN1* gene has now been cloned by transposon tagging and has been found to encode a transmembrane protein with similarity to a group of transporters from bacteria (Fig. 7). It has been suggested that AtPIN1 represents an important component of auxin efflux carrier (26). Alternatively, and equally likely on the basis of currently available genetic evidence, AtPIN1 could act as a regulator of auxin efflux. The Arabidopsis *PIN* gene family consists of eight members, four of which have been characterized in detail in relation to their expression, localization and role in plant development (22). Homologous genes have been found in all other plant species examined, including maize, rice and soybean. To date the proposed auxin efflux catalyst function of AtPIN proteins has not been verified biochemically. Nevertheless, there are several lines of evidence which strongly support such a role for the AtPIN proteins:

1. The AtPIN protein primary sequences and predicted topology suggest a transport function.

The AtPIN proteins share more than 70% similarity and have a closely similar topology - two highly hydrophobic domains with five to six transmembrane segments linked by a hydrophilic region (Fig. 7). Transporters of the major facilitator class display similar topology. Moreover AtPIN proteins were demonstrated to share limited sequence similarity with known prokaryotic and eukaryotic transporters (12, 51, 65, 70, 90).

2. Yeast cells expressing AtPIN2 accumulate less auxin and related compounds.

The only heterologous system used to address PIN transport activity has been yeast cells with an altered ion homeostasis (51). When AtPIN2 is overexpressed in such yeast cells they exhibit enhanced resistance to the toxin fluoroindole, a substance with a limited structural similarity to auxin (51). Other experiments demonstrated that these cells also retain less radioactively labeled auxin (12). The decreased accumulation of labeled auxin or its analogs support an auxin efflux function for AtPIN2 in yeast.

Nevertheless, attempts to demonstrate changes in auxin transport rate in such cells have so far failed, leaving this issue unresolved.

3. AtPIN proteins have a polar distribution in auxin transport-competent cells.

The chemiosmotic hypothesis (see earlier) predicts that auxin efflux carriers are asymmetrically localized in cells and that this polar localization determines the direction of the net auxin flux (76, 79). Coincidence between the polarity of distribution and the known direction of the auxin flux has been demonstrated for several AtPIN proteins. AtPIN1 protein is localized at the basal side of elongated parenchymatous xylem and cambial cells of Arabidopsis inflorescence axes where polar auxin transport occurs in the basipetal direction (26, 70). In contrast, the AtPIN2 protein was polarly localized at the upper side of the lateral root cap and epidermis cells (Fig. 6D; 21, 65) again in accordance with a basipetal auxin stream from the root tip. The AtPIN3 protein localizes predominantly to the lateral side of shoot endodermal cells (Fig. 8B; 23) and the polar localization of AtPIN4 in root tip directs towards columella initials, the site of auxin accumulation (Fig. 6G; 20).

4. Atpin mutants are defective in polar auxin transport.

One of the strongest arguments for the involvement of PIN proteins in auxin transport is a reduction of polar auxin transport in *Atpin* mutants, which directly correlates with loss of *AtPIN* expression in the corresponding tissue. This was demonstrated for basipetal auxin transport in stem of the *Atpin1* mutant and in the root of the *Atpin2* mutant (69, 74).

5. Disruption of AtPIN function cause changes in cell specific auxin accumulation.

Auxin accumulation has been indirectly monitored in Arabidopsis by determining the activity of auxin-responsive constructs such as *DR5::GUS* (81).   Where compared, this seems to correlate well with direct IAA measurements (11, 20). Using this approach, it has been found that changes in cell-specific auxin accumulation in several *Atpin* mutants are correlated with a loss of AtPIN expression in the corresponding cells. Such correlations have been demonstrated for *Atpin2* (*eir1*) mutant roots after gravistimulation (51) and for *Atpin4* mutant roots and embryos (20).   In addition, *Atpin2* (*agr1*) root tips preloaded with radioactively labeled IAA retain more radioactivity than similarly treated wild type roots (12).

6. Phenotypes of Atpin mutants can be phenocopied by auxin efflux inhibitors.

Defects observed in *Atpin* mutants are in processes that are known to be regulated by polar auxin transport and they can be phenocopied by treatment of wild type plants with auxin efflux inhibitors. Examples include the *Atpin1* embryonic and aerial phenotype (69); the defect in root and shoot
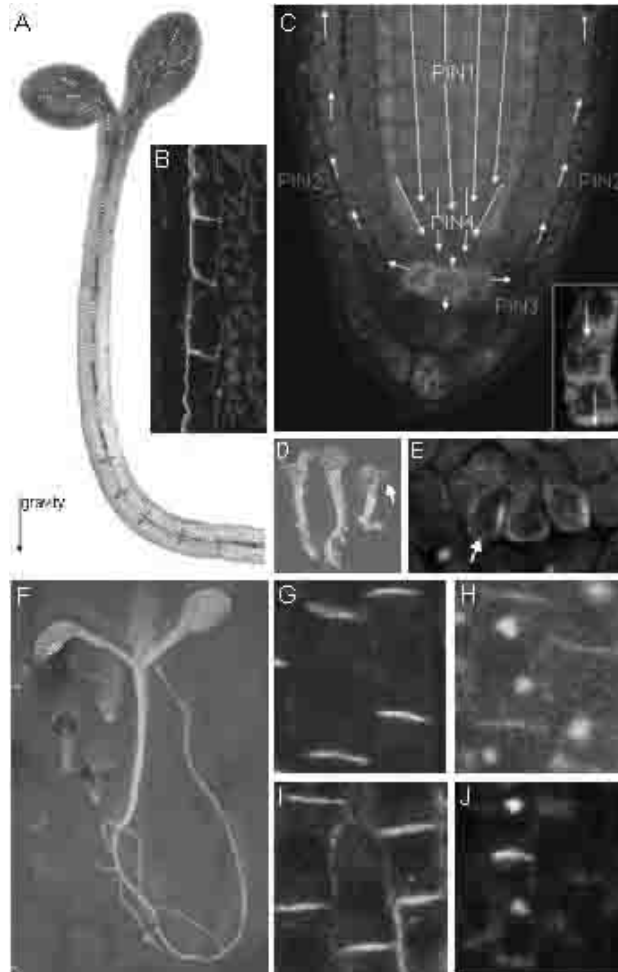
Figure 8 (Color plate on page xxx). A. Elevated DR5 auxin reporter response (blue) in the outer layer of a gravistimulated Arabidopsis hypocotyl. The direction of basipetal (red arrows) and lateral (orange arrows) polar auxin flows is depicted, as well as the non-polar phloem transport of auxin (dashed line). B. Lateral localization of PIN3 in endodermal cells seen in longitudinal section of an Arabidopsis inflorescence. C. Directions of auxin flow derived from the polar localization of various PIN proteins in the Arabidopsis root. PIN3 localization (green) symmetrically around the columella cells is visible. Relocation of PIN3 to the basal side of columella cells after gravity stimulation (inset). D. Seedling phenotypes of *smt1* mutant. Arrows shows triple cotyledon seedling. E. Changes in PIN3 polar localization in the columella of the *smt1* mutant. F. Comparison of wild type Arabidopsis and *gnom* mutant seedlings. G. Polar localization of PIN1 in Arabidopsis root cells. H. Internalization of PIN1 after the BFA treatment. I. Reconstitution of PIN1 polar localization after the BFA removal. J. No internalization of polarly localized PIN1 (red) and internalisation of KNOLLE (green) after BFA treatment in the BFA-resistant GNOM transgenic plant. (A, C from 19; B from 23; D, E from 95; F and J reproduced by courtesy of Niko Geldner).

gravitropism in *Atpin2* and *Atpin3* mutants, respectively (23, 52); the defect in hypocotyl and root elongation in light, in apical hook opening and in lateral root initiation, which have been reported for *Atpin3* mutants (23); and the *Atpin4* root meristem pattern aberrations, which can be also found in seedlings germinated on low concentrations of auxin efflux inhibitors (20).

The data accumulated so far provides an extensive body of evidence to argue that AtPIN proteins are crucially involved in auxin efflux. Nevertheless, the central question of whether PIN proteins represent transport or regulatory components of auxin efflux still remains unresolved. To answer this question, auxin transport assays will have to be developed to establish directly the carrier functions of different the PIN proteins and to determine their substrate specificities, affinities and kinetic properties.

ABC Transporters – More Auxin Transport Proteins?

Recently, a combination of genetic and biochemical approaches has implicated another protein family in auxin transport, namely the so-called multidrug resistance (MDR) proteins, a sub-family of the ATP-binding cassette (ABC) transporters (54). In mammalian system these transmembrane proteins enhance the export of chemotherapeutic substances. Two of them, AtMDR1 and AtPGP1, were originally identified in Arabidopsis as having anion channel-related functions. Nevertheless, the corresponding mutants and double mutants also exhibited phenotypic aberrations consistent with defects in polar auxin transport, such as reduced apical dominance, a lower rate of basipetal auxin transport and also reduced NPA binding. The AtMDR1 and AtPGP1 proteins may transport auxins across both the PM and across intracellular membranes (68; 50). The proteins were isolated by affinity chromatography and were also able to bind NPA *in vitro* or when expressed in yeast cells. In spite of this, membranes prepared from *Atmdr1* mutants still exhibited up to 60% of the NPA binding found in wild type and NPA was able to reduce auxin transport to background levels in the mutant (68). This implies that other NPA-binding protein(s), in addition to MDRs, must be present in Arabidopsis cells (64).

Another ABC transporter, AtMRP5, has also been implicated in auxin biology (25). The corresponding mutant *mrp5-1* displays reduced root growth, increased lateral root formation and higher than normal auxin levels in roots. Thus although much detailed information for ABC transporters is still lacking, especially on their expression and subcellular localization, their direct or indirect connection to the polar auxin transport, and especially to NPA-mediated processes, seems to be established.

Mutations Indirectly Affecting Polar Auxin Transport

In a mutant screen designed to identify components of polar auxin transport based on the fact that auxin efflux inhibitors reduce root elongation, several mutants were isolated in which roots were able to elongate in the presence of

such inhibitors. These mutants were designated *tir* (*transport inhibitor response*; 80), and seven *tir* loci (*tir1 - tir7*) have now been identified. Several of these mutants have been molecularly characterized and this has revealed that the primary defects are in auxin signaling rather than in polar auxin transport. The most relevant for polar auxin transport research is the *tir3* mutant. This displays a variety of morphological defects including reduced elongation of root and inflorescence stalks, decreased apical dominance and reduced lateral root formation. Both auxin transport and NPA binding activity are reduced in the *tir3* mutant (80). It has been suggested that the *TIR3* gene may encode the NBP (see above) or some closely related protein (38). The corresponding gene has been characterized and renamed *BIG* to reflect the unusually large size of the protein it encodes. This has been identified as a protein with several putative Zn-finger domains homologous to the *Drosophila* CALOSSIN/PUSHOVER (CAL/O) protein (32). A defect in CAL/O interferes with neurotransmitter release in *Drosophila*. In Arabidopsis the *big/tir3/doc1* mutations interfere with an effect of auxin efflux inhibitors on subcellular movement of a putative auxin efflux carrier AtPIN1 (32), supporting a role for BIG in vesicle trafficking, although the mechanism of this action is not clarified yet (22, 49, 64).

Another mutant, *pis1* (*polar auxin transport inhibitor-sensitive 1*), isolated in a similar screen, displays a phenotype in many respects opposite to that of the *tir* mutants – it is hypersensitive to some auxin efflux inhibitors (24). This has led to speculation that *PIS1* may encode a negative regulator of the auxin efflux inhibitor pathway. However, lack of molecular data leaves this an open question.

Other genetic work has pointed to a role for phosphorylation in polar auxin transport. A mutant called *rcn1* (*roots curl in NPA*) has been isolated, the roots of which curl in the presence of NPA, in contrast to straight root growth in wild type (27). This mutant has reduced root and hypocotyl elongation and is defective for apical hook formation. The *RCN1* gene was found to encode a regulatory subunit of protein phosphatase 2A. RCN1 may control the level of phosphorylation and thereby the activity of a component involved in polar auxin transport (27; and see below). This possibility has been strengthened by the recent finding that the *rcn* mutant displays enhanced basipetal auxin transport, a phenotype feature which has also been observed in plants treated with a phosphatase inhibitor cantharidin (75). In addition, the *pinoid* (*pid*) mutants in the gene coding for a serine-threonine protein kinase (13) display defects in the formation of flowers and cotyledons, thus resembling plants treated with auxin efflux inhibitors. Moreover basipetal auxin transport in *pid* inflorescences is reduced. The PID protein has been proposed to be involved either in polar auxin transport or auxin signaling. Detailed studies correlating PID expression data with knock out and tissue specific overexpression phenotypes demonstrate that PID action is not cell or organ autonomous and it is sensitive to auxin efflux inhibitors (2). These

data favor the hypothesis that PID is involved in long distance signaling and functions as a positive regulator of auxin transport (2).

Several other Arabidopsis mutants with possible roles in polar auxin transport have been identified in a screen for plants with early developmental aberrations (57). Seedlings of the mutant *monopteros* (*mp*) lack roots and display defects in cotyledon establishment (5). The adult plants show measurable reduction in basipetal auxin transport which, however, might be the result of a reduced vasculature. The *MP* gene codes for auxin response factor 5 (ARF5), a transcription factor, which mediates auxin dependent activation of gene expression (55). It is possible that MP regulates expression of polar auxin transport components. Another mutant with strong seedling phenotype *cephalopod/orc* has been shown to display defects in auxin transport and cell polarity (95). *CPH* encodes sterol methyl transferase 1 (SMT1) – a protein involved in biosynthesis of plant sterols (83). The polar localization of the PIN efflux components is defective in the *cph/orc/smt1* mutant (Fig. 8E), which can account for the defect in polar auxin transport. However, cause and effect in the relationship between PIN localization and cell polarity, and how the defect in sterol membrane composition interferes with this remains unclear.

The connection to polar auxin transport is more clearly characterized in the case of another early development mutant, *gnom* (*gn*), which was isolated from the same screen as *mp* (57). Embryos of *gn* mutants display a variety of aberrations, including defects in apical-basal patterning, and fused or improperly placed cotyledons (Fig. 8F). Most of these defects are reminiscent to defects observed when embryos are cultivated in the presence of polar auxin transport inhibitors (35). The *GN* gene was cloned and the GN protein demonstrated to have guanine nucleotide exchange factor (GEF) activity for small ARF-type GTPases (85). These small GTPases are known to play a role in the control of intracellular vesicle trafficking. It has been shown that *gn* mutant embryos have defects in the correct localization of the polar auxin transport component PIN1 (85). Moreover, recent elegant experiments with GN engineered to be resistant to the inhibitor of vesicle trafficking, BFA (29), show that GN is directly involved in the trafficking of PIN proteins from the endosomes to their polarly localized domain in the PM (Fig. 8J). These observations complete the connection between the polar auxin transport related *gn* phenotype and GN function in vesicle trafficking.

SUBCELLULAR DYNAMICS OF POLAR AUXIN TRANSPORT COMPONENTS

Physiological evidence, which indicates that efflux carrier proteins have a very short half-life in the PM and probably cycle between the PM and an unidentified intracellular compartment, was described above. Subsequent work at the genetic and molecular levels has confirmed the dynamic nature of

the asymmetrically localized PIN proteins. It has been discovered that mutations in the Arabidopsis intracellular vesicle trafficking regulator GNOM, an ARF GEF (see above), interfere with the correct localization of the AtPIN1 protein at the PM during embryogenesis (85). Similarly, chemical inhibition of ARF GEFs by BFA causes the disappearance of PIN1 label from the PM (Fig. 8G) and its intracellular accumulation (Fig. 8H; 30). This reversible BFA effect (Fig. 8I) also occurs in the presence of the protein synthesis inhibitor CHX, thus demonstrating that the internalized AtPIN1 originated from the PM and not as a result of *de novo* protein synthesis (Fig.5).

Together, therefore, physiological and molecular studies have provided good evidence that AtPIN1 cycles rapidly between the PM and a so-called "BFA" compartment, recently characterized as an accumulation of endosomes (29). This model has been corroborated by electron microscopy studies, which detected the homologous PIN3 protein not only at the PM but frequently also in intracellular vesicles (20). The action of drugs that disrupt the structure of the cytoskeleton indicated that AtPIN1-containing vesicles were transported predominantly along the actin cytoskeleton. However in dividing cells, tubulin was also required for correct PIN1 traffic (30).

Recently, experiments using BFA have also demonstrated turnover in the PM of the auxin influx component – the AUX1 protein (34). It seems that the cycling of polar auxin transport components is an essential part of auxin transport since interfering with the cycling process by treatment with BFA, auxin efflux inhibitors, or drugs that depolymerize actin, as well as genetically through mutation of GN, also interfere with auxin efflux and polar auxin transport regulated plant development (16, 30, 60).

Thus previous models of polar auxin transport which envisaged long-lived carriers located asymmetrically in the PM, must now be substantially modified to take account of the new information which demonstrates that auxin carriers (or, perhaps, carrier complexes) are much more dynamic structures than they were originally believed to be. An important unresolved question is the role of AtPIN cycling in polar auxin transport. Several possible scenarios can be conceived:

Firstly, a high turnover of polar auxin transport components would provide the flexibility to allow rapid changes to be made in carrier distribution in the PM and provide a mechanism for the rapid redirection of auxin fluxes in response to environmental or developmental cues (19, 59). This, in turn, would contribute to developmental plasticity underlying adaptive processes such as tropisms, initiation of lateral organs or regulation of meristem activity (see below).

A second possibility is that components of polar auxin transport may have a dual receptor/transporter function (36). In this case cycling might be part of a mechanism for signal transduction and receptor regeneration, as is known for some other kinds of receptors. The issue of dual sensor and

transport functions has been extensively discussed in relation to sugar carriers in yeast cells and in plants (45).

Perhaps the most exciting possibility is that vesicle trafficking itself is a part of the auxin transport machinery and that, in a manner analogous to the mechanism of neurotransmitter release in animals, auxin is a vesicle cargo, released from cells by polar exocytosis (22). In this model PIN localization in endosomes and recycling vesicles would have an entirely new significance. Instead of being PM localized "auxin channels", PIN proteins would mediate the accumulation or retention of auxin in the vesicles in which auxin would be translocated to the corresponding cell pole. Some support of this scenario comes from experiments using anti-IAA antibodies and electron microscopy, in which auxin was found in small vesicles near the PM (84). Moreover, the BIG protein, which is involved in polar auxin transport and PIN1 subcellular trafficking (see above), is a homologue of calossin (32), a protein that mediates vesicle recycling in *Drosophila* during synaptic transmission.

Regardless of how well any of these scenarios (or combinations of them) eventually turn out to fit the true picture, recent advances have made it abundantly clear that gaining an understanding of the cellular mechanisms controlling the subcellular dynamics of the auxin carriers will be crucial if we are to fully understand polar auxin transport.

REGULATION OF POLAR AUXIN TRANSPORT

It might be expected that a system as complex as the one that mediates polar auxin transport will be subject to regulation at a variety of different levels. Polar transport requires the expression of many genes and the synthesis of the corresponding proteins; it requires the targeting of these proteins to defined locations in the cell and at the PM; it involves their metabolic turnover and their cycling; and, in the case of both the transport catalysts themselves and probably also the NBPs, the direct or indirect regulation of their activity. A brief description of some aspects of the regulation of these processes is given here.

The Role of Phosphorylation

The results of physiological experiments indicate that at least some of the processes involved in polar auxin transport are energy-dependent and that phosphorylation/dephosphorylation processes are probably involved in the regulation of the activity of some of the carrier systems. The role of reversible phosphorylation in the regulation of auxin transport has been comprehensively reviewed elsewhere (63) and will be discussed only briefly here. The likely involvement in this regulation of a protein kinase encoded by the Arabidopsis *PINOID* gene (*AtPID*), and of protein phosphatase 2A (PP2A), the regulatory A subunit of which is coded by the *AtRCN1* gene, was described above. Studies of auxin transport in seedling roots of *rcn1* mutants

(in which PP2A activity is reduced) and in cultured tobacco cells treated with various kinase and phosphatase inhibitors, suggest that reversible phosphorylation acts at several different loci in the regulation of polar auxin transport (16, 63). Furthermore, it is likely that reversible phosphorylation and phytotropin action (see below) interact in this regulation. In tobacco cells, kinase inhibitors very rapidly and strongly inhibit auxin efflux without affecting auxin influx (16). In roots of *rcn1* seedlings the sensitivity of acropetal transport to NPA is drastically reduced, suggesting a role for PP2A in NPA function; in contrast, the basipetal return flow of auxin from the root tip is increased substantially and is unaffected by NPA. In this case, PP2A seems to act as a negative regulator of the polar transport system (63, 75).

Nevertheless, it is still not clear how directly, or at what levels the products of *RCN1* and *PID* intervene in the polar auxin transport process and whether they influence the expression and localization, or the activity of the proteins involved. It is also not known how protein phosphorylation-dephosphorylation affects the interaction between the NBP and the auxin efflux carrier system; one recent suggestion is that the reversible phosphorylation loop may mimic or control the activity of the putative, and apparently dynamic, transducing protein proposed to couple the NBP and efflux catalyst action (61, 97). Gaining a clear understanding of the putative role of reversible phosphorylation in the regulation of auxin transport remains a challenge for the future.

The Role of Phytotropins

Phytotropins (such as NPA) have contributed significantly to our understanding of the auxin transport machinery (59, 78). The point has also been made that phytotropins are synthetic compounds with, as yet, no unequivocally identified endogenous equivalent. Some flavonoids have been shown to be competitors of saturable NPA binding to membrane preparations and to inhibit polar auxin transport (9, 41, 78). There is some evidence to show that these endogenous compounds, perhaps together with aminopeptidases, are involved in regulation of auxin transport (66).

How do phytotropins act? At a functional level they simply inhibit auxin efflux, resulting in auxin accumulation by cells. This action appears to require interaction with the NBP, but little is known about the molecular mechanisms involved. Besides, phytotropins may participate in two other cellular processes related to polar transport of auxin: Firstly, some experimental data (73) suggest that NBP is involved in the establishment and the maintenance of polarity of cell division (see below). Secondly, phytotropins have been shown to play a role in auxin efflux carrier cycling through a general inhibitory action on vesicle-mediated traffic to the PM (30, but see72). Studies of the trafficking of PIN1 and other unrelated proteins in Arabidopsis roots have revealed that high concentrations of auxin transport inhibitors interfere with vesicle traffic to and from the PM (30). This has led

to the suggestion that the action of phytotropins on the inhibition of auxin efflux is in fact the result of a non-specific inhibition of protein trafficking, including that of auxin efflux carriers. However, recent observations on BY-2 tobacco cells have revealed that the inhibition of auxin efflux by NPA is much more efficient than the inhibition caused by the well-established inhibitor of protein traffic, BFA, and that BFA, but not NPA, affects some of the intracellular structures related to the trafficking machinery, including the actin cytoskeleton (72). These findings argue against a causal link between a general role of NPA and other phytotropins in vesicle-trafficking and auxin efflux inhibition. Rather, they suggest that a population of auxin efflux catalysts exists which is NPA-sensitive but insensitive to inhibitors of vesicle-mediated traffic.

Generally, the mechanism of regulation of auxin afflux by phytotropins and other inhibitors of polar auxin transport still remains unclear and it is apparent that more work on this topic is needed.

Synthetic Inhibitors of Auxin Influx

In contrast to auxin efflux carriers, work on the physiology of auxin influx carriers has suffered from lack of specific inhibitors. Like efflux carriers, influx carriers may be inhibited by phytotropins such as NPA, but to a very much smaller extent (17). Recently, a promising new group of auxin influx inhibitors of the aryl and aryloxyalkylcarboxylic acid type has been identified (39). Of these, 1-naphthoxyacetic and 3-chloro-4-hydroxyphenylacetic acids have been reported to inhibit auxin influx carrier activity substantially. Both compounds also disrupt root gravitropic responses and in wild type plants mimic the auxin influx carrier mutation *aux1* (71; see above). However, further work in this area is required and we still know nothing about the molecular mechanism by which these compounds inhibit auxin influx carrier function.

Regulation by Auxin and Other Hormones

Auxin itself is needed for the induction of new polar auxin transport pathways, for example during induction and axial development of new vascular tissues (7). A continuous supply of auxin is also necessary to maintain the auxin polar transport system itself. The ability of tissue to transport auxin in a polar manner rapidly decreases following loss of the auxin source, although NPA-sensitive accumulation of auxin is unaffected (33). In both these cases a role for auxin in the regulation of auxin carrier distribution is indicated (59). It remains largely unknown how auxin gradients themselves might establish (or re-establish) new polar transport pathways or change the direction of existing polar transport pathways in response to environmental or internal cues.

Data about the action of other plant hormones on auxin transport are scarce. Much of the currently available information relates to the effects of

ethylene on auxin transport in excised segments of various plant organs, such as maize coleoptiles, bean petioles, cotton stems and petioles (33). In contrast to ethylene, cytokinins have been reported to prevent the decrease in auxin transport in excised tissues in the absence of an auxin source (33). Recently an Arabidopsis mutant was characterised (91) showing alteration in the collaboration between ethylene and auxin, possibly at the level of auxin transport. Nevertheless, the mechanism of the ethylene response is still unclear.

There are some indications that other hormones, including abscisic acid, brassinosteroids and gibberellins, may be involved in the control of auxin transport (88). The physiological importance of mutual control of transport processes for individual phytohormones, including IAA, is obvious; gaining an understanding of the mechanisms by which individual hormones interact at the level of transport remains a major challenge for future research.

AUXIN TRANSPORT AND PLANT DEVELOPMENT

The developmental responses of plants to modifications to the normal patterns of auxin flow make it abundantly clear that auxin transport plays a crucial role in the regulation of development. A bewildering variety of processes may be affected, ranging from cell division, through establishment of polar axes, cell differentiation, pattern formation, histogenesis and organogenesis, to the coordination and integration of biochemical and physiological activities in different regions of the plant body. In some of these processes, auxin transport clearly delivers a signal that acts as a switch, or regulates the progress and rate of a process. In other cases the direction of auxin flow, or, perhaps, tissue gradients in auxin concentration that result from transport, endow auxin transport with both directional qualities and some features characteristic of a morphogen. Here, a few selected examples of the relationship between auxin transport and development will be discussed in order to explore the diversity of this relationship and of the mechanisms probably involved (93).

Cell Division, Initiation of Polarity, and Cell Elongation

Cell division and cell differentiation underpin plant development and in most plant cells differentiation is associated with expansion. The majority of cells in the plant body enlarge by diffuse growth, which is frequently polar in character; i.e. cells tend to elongate preferentially along one axis. Therefore, a mechanism is required to ensure that traffic of secretory vesicles containing material destined for the cell wall and PM is targeted in such a way that it is distributed towards the proper regions of the cell. Auxin seems to be part of the mechanism involved, although to date experimental data relating to this mechanism is fragmentary and incomplete.
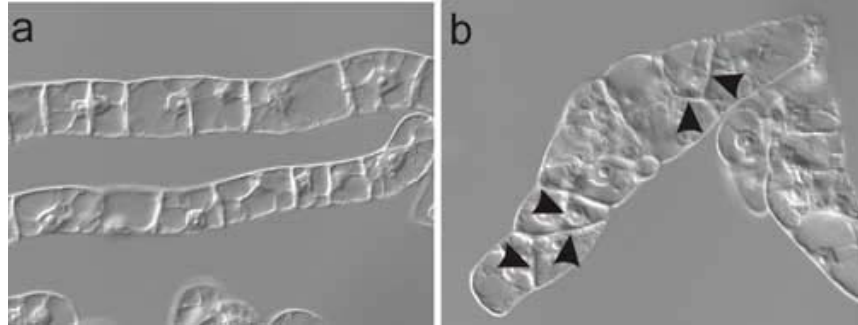
Figure 9. Effect of 1-N-naphthylphthalamic acid (NPA) on the polarity of cell division of suspension-cultured cells of *Nicotiana tabacum* L. line VBI-0. **a**. Cells grown in control medium, day 9. **b**. Cells grown in control medium supplemented with NPA (final concentration 10 μM), day 9. Note abnormal cell division planes (black arrows). Scale bars = 50 μm. (Modified from 73).

Attempts to explore the role of polar auxin transport in the initiation of cell division and in the establishment of polarity at the single cell level have been hampered by lack of a suitable model system. The wide diversity of cell types within even a relatively simple structure, such as the seedling root of Arabidopsis, makes it almost impossible to investigate either biochemical or some cytological aspects of auxin transport at the cell level in a meaningful way. Because of this, attention is increasingly focussing on the development of cell suspension culture systems in which individual cells separate easily and in which the stages of cell development can be readily synchronised. Although so far no such system is available for Arabidopsis, recent studies suggest that some well-defined tobacco cell lines (28) may provide a suitable alternative model system in which to conduct parallel studies of the genetic, cellular and biochemical aspects of polar auxin transport.

Immobilised cultured cells of *Nicotiana tabacum* L., derived from leaf mesophyll protoplasts, have been used to study cell elongation in initially spherical cells after the addition of NAA (92). No polar ion fluxes, typical of those found in tip growth, were detected, indicating that the positional information required to establish a polar axis was delivered by a different signal, possibly auxin itself. A model was proposed whereby an auxin flux initiated a signal cascade that resulted in a reorientation of microtubules which guided deposition of cellulose in the cell wall. The resulting modification of the cell wall mechanics promoted elongation growth.

Changes in auxin (NAA) accumulation and auxin efflux carrier activity during the growth cycle of another model tobacco cell line have also been studied in detail (73). Although the level of NAA accumulation remained relatively stable over a subculture period, the sensitivity of cells to NPA changed markedly and reached a maximum at the onset of division. Treatment of cells with NPA delayed the onset of cell division but did not

prevent it. However, when cell division commenced a significant proportion of the NPA-treated cells exhibited loss of cell polarity and abnormal orientation of cell division (73; Fig. 9). These observations, together with data suggesting that the NBP may affect the localization of auxin efflux carriers (32, 49), indicate that in tobacco cells the NPA-sensitive directed traffic of auxin efflux carriers to the specific regions at PM may regulate the orientation of cell division (73).

An interesting link between cell polarity and the sterol composition of cell membranes has been described recently (95) from *cephalopod/orc* mutants of Arabidopsis (see above). In the mutants the PIN proteins, but not AUX1, are mislocalized and the mutants show a wide range of polarity defects. As neither membrane fluidity nor vesicle trafficking seems to be impaired, mutation of the *CPH/ORC/SMT1*gene may disrupt the docking of proteins to specific membrane microdomains or lipid rafts (95). Together the data reveal the importance of a balanced membrane sterol composition for auxin efflux and for promoting cell polarity in Arabidopsis.

Auxin Transport and Vascular Development

The role of auxin in the induction of vascular tissues is covered in depth in chapter E2 and treatment of the topic here will be confined to a brief examination of the possible part played by polar auxin transport itself in determining the axes and pathways of vascular development.

Vascular differentiation normally takes place along well defined pathways initially laid down in the embryo as provascular strands of narrow, elongated cells early in the development of an organ (6). Although usually predictable, the ease with which new axes of vascular development may arise following wounding and/or hormone application demonstrate considerable flexibility in vascular patterning (6, 82). It is now clear that vascular differentiation occurs along axialized (canalized) auxin flows. These may be generated naturally by auxin originating in the apical regions of developing organs, including the developing embryo itself, or experimentally by organ removal and replacement with exogenous auxin (6, 82). Canalized auxin flows may themselves by mediated by PIN proteins (see earlier). In auxin transport mutants or in plants treated with polar auxin transport inhibitors, abnormal patterns of vascular development frequently occur. For example, following application of increasingly high concentrations of NPA to Arabidopsis seedlings, the normal pattern of rosette leaf vein formation becomes increasingly disrupted (56). This observation is consistent with the canalization hypothesis (82,), which postulates that through a feedback mechanism, an initially weak polar auxin flow through a file of cells renders them more conductive and increases polar auxin flow. This will tend to "drain" surrounding tissues until a point is reached at which the concentration of auxin in the conducting cells exceeds some critical threshold required for vascular differentiation. The postulated feedback mechanism is unknown,

but the ability of auxin to stimulate the accumulation of H^+-ATPase protein at the PM and for BFA to inhibit auxin-mediated growth and secretion of cell wall proteins leads to speculation that auxin may promote Golgi-dependent traffic of components of the polar auxin transport system itself (59). Precisely how the canalized auxin flow regulates the differentiation of vascular elements remains uncertain, but the transported auxin probably up-regulates the activity of ARF proteins such as MP by removing their inhibition by AUX/IAA proteins such as BODENLOS (BDL). This enables the ARFs to interact with appropriate auxin responsive elements and activate genes involved in early axialization and development of provascular strands (93).

Tropisms

Tropisms are permanent changes in the direction of growth of an organ caused by differential growth rates on either side of the organ in response to a directional environmental stimulus such as light (phototropism) or gravity (gravitropism). The Cholodny-Went hypothesis (94) proposed that the different growth rates on either side of an organ resulted from an unequal distribution of auxin between its two sides in response to the stimulus. To explain the different directions of root and shoot growth in response to a stimulus (i.e. roots are positively gravitropic, shoots negatively gravitropic) the hypothesis proposed that in shoots a high auxin concentration promoted, and in roots inhibited, cell elongation resulting in bending responses in opposite directions. Many experiments have demonstrated a differential distribution of auxin or of auxin response following stimulation and these have been correlated with the change in growth rate and the bending response (Fig. 8A). Because auxin efflux inhibitors interfere with the asymmetric distribution of auxin and tropisms, polar auxin transport has been implicated as the process by which asymmetric auxin distribution is achieved (19). Radial polar transport of auxin has been proposed to facilitate the exchange of auxin between the main basipetal stream in shoot vasculature and peripheral regions where control of elongation occurs. Molecular support for these proposals was obtained following the identification of auxin efflux component PIN3. The PIN3 protein is involved in hypocotyl and root tropisms and is localized in shoot at the lateral side of endodermal cells (Fig. 8B), where it is perfectly positioned to regulate radial auxin flow (23). Since the lesions in tropic responses in *pin3* mutants are rather subtle, other PIN proteins may functionally replace PIN3 (23).

In roots gravitational stimuli are perceived in the root cap but the growth response occurs in the elongation zone where elevated auxin levels on the lower side inhibit growth, causing downward bending. Results have shown that the initial response is a lateral redistribution of auxin towards the lower side in the root cap itself, from where it is transported basipetally to the elongation zone (Fig. 8C; 74, 81). Both efflux and influx components of

polar auxin transport are involved in these processes. Localization and mutant studies suggest that the putative influx carrier AUX1 facilitates auxin uptake into the lateral root cap and epidermis region and the efflux regulator PIN2 mediates directional translocation towards the elongation zone (Fig. 8C; 19)

How is polar auxin transport linked to the perception of a stimulus, such as gravity? Gravity is perceived by sedimentation of starch containing organelles (statoliths) in the columella region of the root cap and in shoot endodermis ("starch sheath"). The presence of PIN3 in these cells suggested the possibility that gravity perception and auxin redistribution are coupled *via* PIN3. This possibility has now been tested in gravistimulated Arabidopsis roots. During normal downward growth of the root the majority of PIN3 is located symmetrically at the columella cell boundaries. However, within two minutes of gravistimulation, the position of PIN3 changes and it becomes relocated towards the new lower end of the cells (Fig. 8C, inset). Thus PIN3 is ideally placed to mediate an auxin flow towards the lower side of root (Fig. 10). Interestingly, AUX1 is also localized in columella cells, both at the PM and internalized. One possibility is that AUX1 mediates auxin influx into the columella after gravistimulation, thereby creating a temporary pool of auxin needed for asymmetric redistribution in a PIN3-dependent pathway.

But how is PIN3 so rapidly relocated after the perception of a stimulus? The answer may lie in the rapid cycling of PIN proteins along the actin cytoskeleton, as already discussed above. Such a mechanism would provide the necessary rapidity and flexibility of response, which is unlikely to be achieved through *de novo* protein synthesis and targeting.

An important question remains for future investigation in relation to

Figure 10. Model of root gravitropism. Auxin is provided to the root tip through the stele, is laterally distributed symmetrically from the columella and is basipetally transported through the lateral root cap and epidermis to the elongation zone of the root. After reorientation of the root, statoliths in the columella sediment to the lower side of the cells (inset), PIN3 auxin transport proteins are relocated and facilitate auxin transport to the lower side of root. From there auxin is transported to the elongation zone, where it inhibits elongation resulting in downward bending.

gravitropism: How is the sedimentation of statoliths connected to PIN3 relocation? Several observations suggest that the actin cytoskeleton reorganizes during statolith sedimentation. Thus, the actin dependent intracellular traffic of PIN3 could be redirected along the sedimentation routes and PIN3 would preferentially accumulate at the lower side of the cell.

However, so far there is only indirect evidence that it is indeed PIN3 relocation that mediates auxin redistribution. The presence of both statoliths and PIN3 in the shoot endodermis suggests that a similar mechanism involving relocation of PIN3 and/or other PIN proteins also operates during shoot tropisms - but this too remains to be demonstrated unequivocally.

Another important issue concerns phototropism. Although it seems to be well established that PIN-dependent asymmetric auxin distribution also underlies this process, the mechanism by which light direction acts to influence lateral redistribution of auxin remains unknown and is a topic for future investigations (63).

Patterning - Polar Auxin Transport and the Maintenance of a Morphogen Gradient

The role of auxin and polar auxin transport in roots is not restricted to growth responses. Exogenous manipulation of auxin levels as well as analysis of mutants impaired in auxin signaling, have demonstrated a role for auxin in the regulation of patterns of cell division and differentiation (Fig. 11; 43, 80) and have renewed the debate about a possible role for auxin as a morphogen (81). From an animal standpoint, rigorous definition of a morphogen requires: (i) the formation of a stable concentration gradient of the compound in question; (ii) that the compound itself should directly "instruct" the responding cells (and not work through another signalling pathway); and (iii) that the magnitude of the response (of a cell) should depend on morphogen concentration. Evidence is accumulating, albeit mainly indirect, to demonstrate that auxin shares some of these properties. Chemical analysis has revealed a graded distribution of free auxin in Scots pine stem (89) and along the *Arabidopsis* root (11). These findings have been corroborated indirectly by the use of auxin response reporters (*DR5::GUS)* to indirectly visualize auxin gradients within the root meristem (Fig. 11), forming a maximum in the columella initial cells (20, 81). Intriguingly, the auxin efflux component PIN4 is expressed in this region and asymmetrically positioned towards cells with increased *DR5::GUS* response (Fig. 6G). Moreover *pin4* mutations, as well as chemical inhibition of auxin efflux, disrupt the spatial pattern of distribution of the *DR5::GUS* response. These findings suggest that efflux-driven auxin transport actively maintains the auxin gradient. The observed changes in the auxin gradient are accompanied by various patterning defects and correlate well with changes in cell fate. Thus it appears that efflux-dependent auxin distribution is linked with cell fate acquisition and thus correct meristem patterning. However, as yet we know

too little about auxin gradient perception and downstream signalling to be able to pinpoint the direct effect on instructed cells. The emerging model of active, regulated, polar transport-driven auxin gradients differs from the classical view of animal morphogens, which are supposed to freely diffuse from a localized source. Interestingly, in the animal field too, the morphogen concept is being revised, since the gradients of classical morphogens such as Decapentaplegic or Wingless appear to be actively maintained by vesicular trafficking (18), thus showing similarities to GNOM-regulated polar auxin transport (see earlier).

It seems that auxin and its graded distribution also plays a role in patterning of embryos. Exogenous manipulation of auxin homeostasis in *in vitro* cultured *Brassica* embryos demonstrated a role for polar auxin transport in the establishment of the apical-basal axis and in cotyledon separation (35).
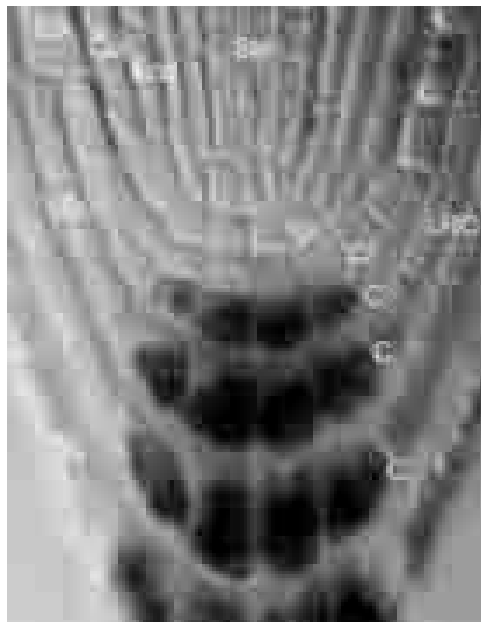

Figure 11. Arabidopsis root meristem pattern. The quiescent centre (QC) in the middle is surrounded by undifferentiated initials, including the columella initials (Ci), which give rise to columella (C), where starch containing statoliths occur (stained here by lugol). Other differentiated cell types such as epidermis (Epi), cortex (Cor), endodermis (End), stele (Ste) and lateral root cap (LRC) are indicated. This regular and invariant pattern correlates with the auxin gradient, which displays its maximum in the columella initials. (Reproduced with permission from 19).

Genetic studies with mutants impaired in auxin signalling, such as *mp*, *bdl* and *auxin resistant 6* (*axr6*), as well as with the *gn* mutant impaired in proper localization of PIN proteins, further strengthen this hypothesis. Possible auxin distribution and polar auxin transport routes in embryos can be indirectly inferred from the polar localization of the PIN1 and PIN4 proteins, which are expressed in embryos (Fig. 6F). In such a model, auxin would be transported through the outer layers towards the future cotyledon tips and then through the centre towards the embryo base (corresponding to the location of the *DR5::GUS* response maximum) and further exported through the suspensor (Fig. 12). Nonetheless there is still a substantial amount of experimental work needed to verify this model and to clearly pinpoint the role of auxin in embryo development. So, despite the fact that we can not with our present knowledge decide whether auxin rigorously

Figure 12. Model of auxin transport and distribution during Arabidopsis embryogenesis. Most auxin is transported in a PIN1- and PIN4-dependent manner to the basal part of the embryo, where the root meristem is specified. *mp*, *bdl*, *gn* and *pin4* show defects in root pole establishment. Additional sites of auxin accumulation appear at the tips of forming cotyledons. *mp*, *gn*, *pid* and *pin1* are defective in cotyledon establishment. Presumed sites of auxin accumulation are indicated by shading (grey). Arrows indicate routes of auxin efflux. Proteins involved in embryo patterning and related to auxin transport (encircled) or responses are depicted.

fulfils the definition of a morphogen, it certainly meets the most important criteria of it – it exhibits a graded distribution and it is involved in patterning processes. However the important issue of the interpretation of auxin gradients remains a challenge for future research.

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A

B

C   PIN1

PIN2   PIN2

PIN4

PIN3

gravity

D   E

F   G   H

I   J