Auxin Biosynthesis and Homeostasis in *Arabidopsis thaliana* in Relation to Plant Growth and Development

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Abstract

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The auxin indole-3-acetic acid (IAA) is a growth regulating substance important for many developmental processes during the life cycle of plants. The papers presented in this thesis address different aspects of IAA biosynthesis, metabolism and transport. The model plant *Arabidopsis thaliana* was used for most of the studies, but some studies were also performed on Scots pine (*Pinus sylvestris*). We developed very sensitive and selective mass spectrometric analytical techniques that made it possible to perform tissue specific IAA quantification and IAA biosynthesis rate measurements on small amounts of plant tissue.

We observed that seeds utilised stored IAA (in the form of ester- and amide-linked conjugates) for elongation growth during the initial germination phase. IAA biosynthesis and catabolism were initiated later in the germinating seedling, and these processes appear to be tightly regulated in order to maintain IAA homeostasis in the developing tissues. High concentrations of IAA were observed in young developing leaves and tissues with high rates of cell division. Perturbation in the IAA concentration within the leaf lowered leaf expansion, and feedback inhibition of IAA biosynthesis was observed after NPA treatment to block polar auxin transport. The youngest developing leaves exhibited the highest IAA biosynthesis rates, but all parts of young seedlings, including the root, showed IAA synthesis capacity.

We demonstrated that transport of IAA from the shoot to the root is essential for the emergence of lateral root primordia, and that a basipetal IAA gradient is present in the root tip. We also showed that this gradient is probably generated by the cellular localisation of auxin influx and efflux carriers, directing the flow of auxin coming from the aerial parts of the plant to specific cell types within the root tip. In addition to IAA synthesised in the shoot and then transported to the root system *via* polar auxin transport and/or transport in the phloem, we demonstrated that a source of IAA is located within the root tip.

Key words: auxin, biosynthesis, feedback inhibition, metabolism, homeostasis, cell division, leaf development, lateral root development, polar auxin transport, phloem transport, gradient.

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Lätta uppmjukningstankar

Att tänka fritt är stort.
Att tänka om är större.
Att tänka först är störst.
Att tänka smått är gott.
Att tänka snett är lätt.
Att tänka galet är idealet.
Att tänka sakligt är smakligt.
Att tänka lagligt är behagligt.
Att tänka snuskigt är ruskigt.
Att tänka klart är smart.
Att tänka skumt är dumt.
Att tänka fel är fel.
Att tänka löjligt är möjligt.
Att tänka genialt är fatalt.
Att tänka luftigt är förnuftigt.
Att tänka mycket sakta är inte att förakta.

Att tänka fritt är stort.
Att tänka stort är stort.
Men inte alltför stort.

Immanuel Kantliga tankar
och andra ofantliga tankar
dom får inte rum i huvet
du vet.

Tage Danielssons Tankar från roten, 1974

Contents

Appendix

List of papers

This thesis is based upon the following papers, which will be referred to in the text by the corresponding Roman numerals.

I. **Ljung, K.*, Östin, A.*, Lioussanne, L. and Sandberg, G.** (2001). Developmental regulation of indole-3-acetic acid turnover in Scots pine seedlings. *Plant Physiol.* **125**, 464-475.

II. **Ljung, K., Bhalerao, R.P. and Sandberg, G.** (2001). Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *Plant J.* **28**, 465-474.

III. **Bhalerao, R.P.*, Eklöf, J.*, Ljung, K.*, Marchant, A., Bennett, M. and Sandberg, G.** (2002). Shoot derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. *Plant J.* **29**, 325-332.

IV. **Ljung, K. and Sandberg, G.** (2002). Auxin biosynthesis in *Arabidopsis* root apical tissue. (Manuscript).

V. **Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, K. and Bennett, M.** (2001). Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. *Genes Dev.* **15**, 2648-2653.

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

* To be considered joint first authors.

Introduction

Background

The development of a seed to a fully-grown plant while tightly regulated, is a plastic process. The basic architecture of the plant is laid out in the embryo, and during plant growth and development the primary and secondary meristems continuously give rise to new leaves and branches, flowers, roots and stem tissue. During germination and subsequent growth, a plant must be able to adapt to a range of environmental conditions in order to develop and reproduce successfully. This holds for both annual plants, such as the small weed *Arabidopsis thaliana*, as well as for 3000-year-old *Sequoia giganteum* trees. Plants can respond rapidly to changes in environmental factors such as temperature, light intensity and nutrient supply. Environmental signals induce responses in diverse groups of cells and tissues *via* specific intrinsic signal transduction pathways that influence cell division, cell expansion and cell differentiation processes, and thus adjust growth and development patterns as appropriate. A group of small organic molecules that affect physiological processes at very low concentrations (Davies, 1995; Leyser, 1998) referred to as plant hormones or plant growth regulating substances, play important roles in the developmental processes described above. Although there are some similarities between animal and plant hormones in their chemical structures and modes of action, plant hormones have unique characteristics in the way they control growth and development. Among the major classes of plant hormones (auxins, cytokinins, gibberellins, abscissic acid, brassinosteroids and ethylene), auxins and cytokinins have the distinction of being required for viability since no loss of function mutants have been described for these hormone classes. They are also needed from embryogenesis throughout the whole life cycle of the plant. Recent advances in biochemistry, analytical chemistry and molecular biology, and the use of the plant *Arabidopsis thaliana* as a common model system, have dramatically increased our knowledge of developmental processes in plants. There is growing insight into how plant hormones affect growth and development and in some cases the specific biochemical mechanisms that they regulate, but many processes are still only vaguely understood.

Many developmental processes are influenced by auxin, including embryo development, the differentiation of leaves and vascular tissue, primary and lateral root development, apical dominance, fruit development and tropisms. How a simple substance is able to influence such diverse processes has been a puzzle to plant physiologists for many years, but important discoveries made in recent years are beginning to increase our knowledge about auxin signalling. It has been proposed, for instance, that auxin can act as a morphogen during plant development (Jones, 1998; Sabatini *et al.*, 1999). Auxin has been shown to be distributed in spatial gradients in plant tissues, e.g. the vascular cambium (Uggla *et al.*, 1996). The formation of this gradient is believed to be dependent on polar auxin transport, and it was proposed that auxin regulates the developmental fate of cells in the cambial region. Another important discovery is the importance of controlled protein degradation in auxin signalling (Gray and Estelle, 2000; Leyser, 2001; Dharmasiri and Estelle, 2002). This

can affect the expression of numerous downstream genes in the signal transduction pathways. Which pathways that are affected will depend on the developmental state of the individual cells, leading to different responses in different cell types.

Auxin biosynthesis and metabolism

The major naturally occurring auxin is indole-3-acetic acid (IAA). It is physiologically active in the form of the free acid, but can also be found in conjugated forms in plant tissues. The redundancy in the biosynthetic pathways leading to IAA has made it difficult to elucidate the different enzymatic steps in IAA synthesis. However, an increasing amount of data points to IAA being synthesised by at least two different pathways (reviewed in Normanly and Bartel, 1999; Ljung *et al.*, 2002; Bartel *et al.*, 2002), in one of which tryptophan (Trp) is the precursor, while indole-3-glycerol phosphate (IGP) is the putative precursor in the other. How these different pathways play a role in plant development is not yet understood. As well as being a precursor in Trp-dependent IAA biosynthesis, tryptophan is also a precursor in the biosynthesis of indole glucosinolates (reviewed in Celenza, 2001), and some of the mutants that have been found to contain high amounts of IAA, such as *rnt1/sur2* and *bus1/sps*, are actually defective in different enzymatic steps of glucosinolate biosynthesis. Tryptophan is believed to be synthesised in chloroplasts and plastids, and it has been suggested that IAA can be synthesised both in chloroplasts and in the cytoplasm (Nonhebel *et al.*, 1993; Sitbon *et al.*, 1993). However, this is still a matter of debate since the subcellular locations of the majority of the different enzymes involved in IAA biosynthesis are not known (Bartel *et al.*, 2001). It is very likely that regulation of IAA biosynthetic rates plays an important part in the overall control of IAA pool sizes, possibly by feedback inhibition of specific steps in the pathway(s). Additionally, a plant can control the pool size of IAA by conjugation (either reversible or irreversible), compartmentation, catabolism and transport. Newly synthesised IAA can act either directly on the cell where it has been produced, or it may be transported out of the cell *via* auxin efflux carriers to other cells and tissues. The major IAA catabolites and conjugates have been identified in *Arabidopsis* (Östin *et al.*, 1998), but little is known at present about how the formation of these substances is regulated or even the identity of the catabolic and conjugation enzymes. **Figure 1** illustrates the different inputs to and outputs from the pool of free IAA in a cell. Possible regulatory mechanisms that influence auxin biosynthesis, transport and signalling are also outlined.

Auxin perception and signal transduction

So far, only one good candidate for an auxin receptor has been identified, namely ABP1 (auxin binding protein 1). ABP1 has been identified in several plant species, including *Arabidopsis*, where it is encoded by a single gene (Timpte, 2001). Recent studies have demonstrated that ABP1 is essential to embryo development (Chen *et al.*, 2001a) and that it has an important role in cell expansion (Jones *et al.*, 1998;

Chen *et al.*, 2001b). The protein has a C-terminal KDEL sequence, directing it to the endoplasmatic reticulum (ER), but a small proportion appears to be located at the plasma membrane (PM), where it probably interacts with a PM-bound docking protein and/or an ion channel, thereby transmitting the auxin signal to the cell interior. The function of the ER-localised ABP1 is unknown, but a role in directing cell wall material to expanding cell walls by vesicle trafficking has been suggested (Timpte, 2001).

The auxin response involves the rapid (i.e. minutes to a few hours) induction of a number of genes, including members of the *SAUR*, *GH3* and *Aux/IAA* gene families (reviewed in Abel and Theologis, 1996). Induction of many of these genes is independent of *de novo* protein synthesis, and the promoters of these genes contain specific auxin-responsive *cis*-acting elements (AuxREs) that can interact with transcription factors (auxin response factors, ARFs) and regulate gene expression in an auxin-dependent manner (Guilfoyle *et al.*, 1998; Reed, 2001). The Aux/IAA proteins have nuclear-localisation sequences in addition to four conserved regions (domains I, II, III and IV) that are important for protein/protein interactions and protein stabilisation. The ARFs contain regions homologous to domains III and IV of the Aux/IAA proteins, and also a DNA binding domain. Domains III and IV of the Aux/IAA and ARF proteins can mediate homo- or hetero- dimerization between these proteins, leading to activation or repression of specific genes. *Arabidopsis* has 25 genes encoding Aux/IAA proteins and 23 genes encoding ARFs, so the number of possible interactions is quite large. The *Aux/IAA* genes show tissue-specific expression, and several mutations in *Aux/IAA* and *ARF* genes have been found that give rise to auxin-related phenotypes (Reed, 2001). Light and auxin signalling are probably linked (Colón-Carmona *et al.*, 2000; Hsieh *et al.*, 2000). For instance, Aux/IAA proteins have been shown to interact with, and to be phosphorylated by, phytochrome A *in vitro* (Colón-Carmona *et al.*, 2000). In addition, there is growing evidence for the involvement of the ubiquitin-proteasome pathway in regulating auxin responses, probably *via* controlled degradation of AuxIAA proteins (reviewed in Gray and Estelle, 2000; Leyser, 2001; Rogg and Bartel, 2001). Degradation of Aux/IAA proteins is believed to be necessary for normal auxin signalling, and some *Aux/IAA* mutants contain higher levels of the protein than wild type plants, indicating that protein stabilisation is affected (Reed, 2001).

Mitogen-activated protein kinases (MAPKs) may also play a role in auxin signal transduction (Hirt, 1997; Zwerger and Hirt, 2001). MAPKs can regulate gene expression by phosphorylation of specific transcription factors. MAPKs are themselves activated by phosphorylation, by MAPKKs, which in turn are activated by MAPKKKs (also by phosphorylation). A tobacco MAPKKK (NPK1) was recently shown to activate a MAPK cascade, leading to suppression of the *GH3* promoter (an early auxin-response gene; Kovtun *et al.*, 1998). Overexpression of *NPK1* led to low germination rates and defects in embryo development.

A molecular link between oxidative stress, auxin response and cell cycle regulation has been established, suggesting cross-talk between these signal transduction pathways (Hirt, 2000; Kovtun *et al.*, 2000). A possible interaction between auxin and calcium/calmodulin (Ca^{2+}/CaM) signalling is supported by the discovery

of a CaM-binding protein in maize encoded by a gene with sequence similarity to *SAURs* (small auxin up-regulated RNAs) (Yang and Poovaiah, 2000). The *ZmSAUR1* gene was induced by auxin within 10 min, and Ca^{2+}/CaM was believed to regulate ZmSAUR1 post-translationally. Involvement of Ca^{2+}/CaM and SAURs in the regulation of cell elongation was postulated.

There is substantial evidence that the different hormone signal transduction pathways interact with each other, and that this cross-talk influences plant growth and development (Ross and O´Neill, 2001; Swarup *et al.*, 2002). Ross *et al.* (2000) showed that IAA up-regulates a gibberellin 3β-hydroxylase (*LE, PsGA2ox1*), promoting elongation in pea stem internodes. Interaction between auxin and cytokinin signalling has been extensively investigated, showing that these hormones interact in a complex manner (Coenen and Lomax, 1997). There are also indications of cross-talk between auxin and jasmonate signalling (Sasaki *et al.*, 2001) and auxin and ABA signalling (Suzuki *et al.*, 2001).

One of the first detectable responses to auxin is the hyperpolarization of the plasma membrane (Macdonald, 1997). This response has a lag time of only a few minutes and involves the activation of H^+-ATPases at the plasma membrane, leading to increased rates of proton pumping and acidification of the cell wall and apoplast. It has been suggested that this decrease in pH can activate specific enzymes that loosen the cell wall, causing cell expansion. Hyperpolarization of the plasma membrane can be inhibited by antibodies to either ABP1 or to H^+-ATPases.

The auxin signal transduction pathways lead to the induction of a number of early and late auxin-response genes and other cellular processes that are only just beginning to be understood. The induction of specific genes, e.g. genes encoding expansins, H^+-ATPases and K^+ ion-channel proteins, is believed to be important to the process of cell expansion. Cell division is also believed to be under hormonal control, with both auxins and cytokinins as important factors (den Boer and Murray, 2000). Most work done in this field of research has focused on tissue cultures and cell suspensions and it has proved difficult to verify the results obtained from these *in vitro* systems with observations obtained *in planta*.

Auxin transport

Auxin is unique among the plant hormones in that it is transported through plant tissues in a polar fashion. This polar auxin transport (PAT) is mediated *via* specific influx and efflux carriers, located at the plasma membrane (for reviews see Morris, 2000; Muday and DeLong, 2001; Friml and Palme, 2002). In addition to PAT, auxin can also be transported *via* the phloem (Baker, 2000). IAA unloaded from source tissues into the phloem should theoretically dissociate to its charged form. Due to the high pH of the phloem sap (around 8) IAA will remain unprotonated and thus trapped in the phloem until encountering a specific transporter. IAA could be transported in the phloem together with photoassimilates, and then be unloaded into sink tissues such as roots or developing leaves (Ruiz-Medrano *et al.*, 2001). Phloem transport is much faster than PAT (around 1 cm/min or more compared to 0.5-2 cm/hour),

but the relative contributions that PAT and phloem transport make to the auxin pools in different tissues are not known. It is likely that these two transport streams of auxin serve different purposes during distinct phases of plant growth and development.

The investigation of mutants affected in PAT and the use of auxin transport inhibitors have greatly increased our knowledge of how auxin is transported and the significance of PAT to plant growth and differentiation. The *aux1* (*auxin resistant 1*) mutant was identified in a genetic screen for mutants resistant to exogenous auxin and was found to have abolished root gravitropism (Picket *et al.*, 1990; Marchant *et al.*, 1999). The wild-type gene corresponding to the mutant loci was cloned and shown to encode an amino acid permease-like gene (Bennett *et al.*, 1996) and *AUX1* was found to be highly expressed in primary and lateral root tips and in the shoot apical meristem (Marchant *et al.*, 2002). Three *AUX1*-related genes (termed *LAX1-3* (*like-AUX1*)) have also been found in *Arabidopsis* (Swarup *et al.*, 2000). The function and expression patterns of these genes are not known, but it is likely that they are also involved in auxin transport.

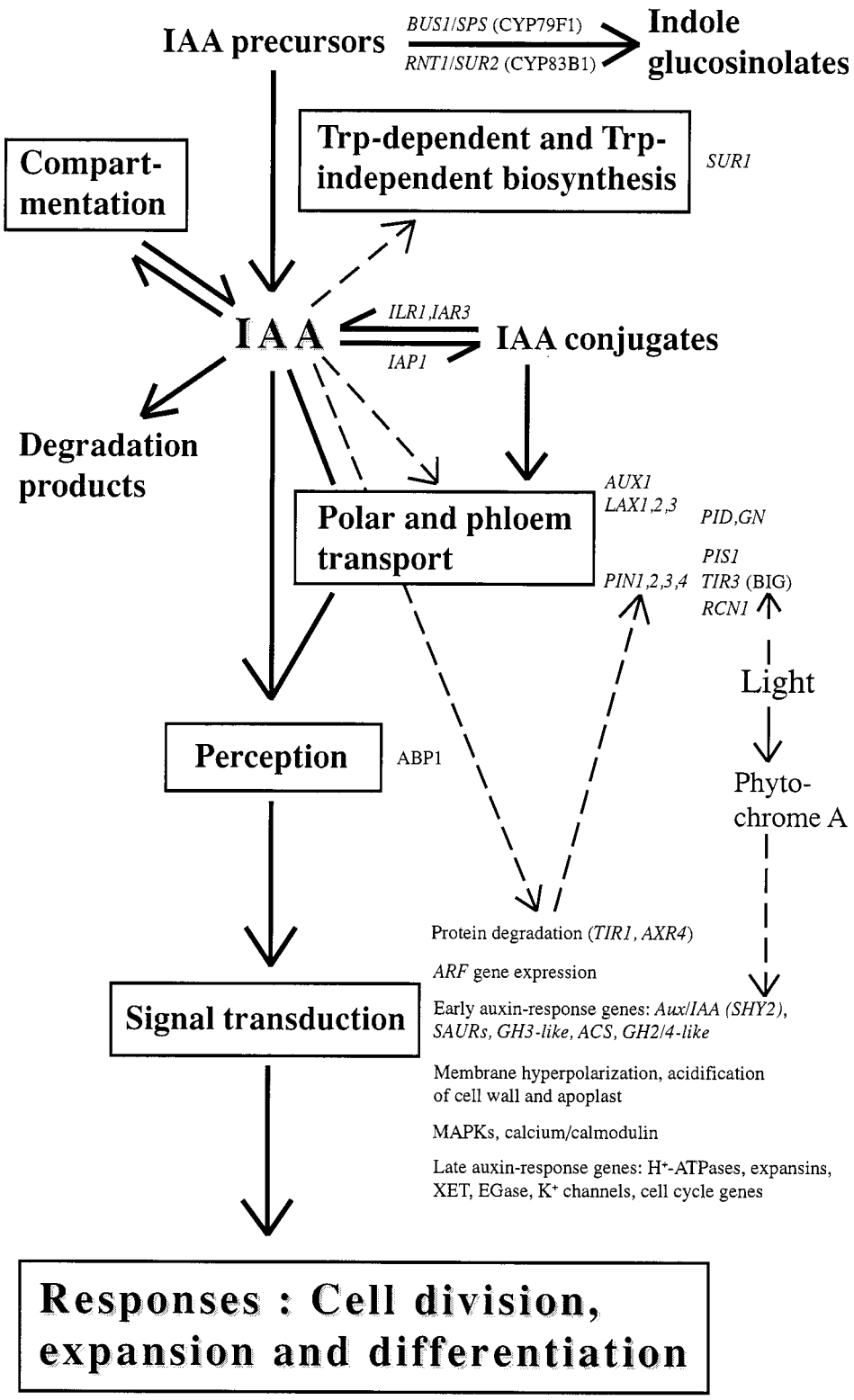
The first auxin efflux carrier genes to be cloned were the *PIN* (*pin-formed*) genes, *AtPIN2* (also known as *AGR1/EIR1*) and *AtPIN1* (Palme and Gälweiler, 1999; Morris, 2000). These genes have tissue-specific expression patterns and the proteins they express show a polar distribution at the plasma membrane of auxin transport-competent cells (Gälweiler et al., 1998; Müller, 1998). Two other *PIN*-genes have recently been characterised, namely *AtPIN3* (Friml *et al.*, 1999; Friml *et al.*, 2002) and *AtPIN4* (**Paper VI**). Based on sequence similarity, at least 14 *PIN1*-related genes are believed to exist in the *Arabidopsis* genome.

Three mutants have recently been characterised that show phenotypes typical of plants with disturbed PAT (i.e. changes in root growth responses and changes in the sensitivity to PAT inhibitors). The *tir3* (*transport inhibitor response*) mutant shows a reduction in PAT, reduced apical dominance and lack of lateral roots. The *TIR3* gene encodes a very large calossin-like protein called BIG (Gil *et al.*, 2001) that seems to be essential for proper localisation of the auxin efflux carrier to the plasma membrane. *N*-1-naphthylphthalamic acid (NPA) inhibits PAT by binding to a protein associated with the auxin efflux carrier, and BIG is a putative NPA-binding protein. The discovery that *tir3* is allelic to the light response mutant *doc3* (Li *et al.*, 1994) suggests a link between light signalling and PAT. Investigation of the *rcn1* (*roots curl in NPA*) mutant has led to the suggestion that protein phosphorylation may regulate auxin efflux (Rashotte *et al.*, 2001). *RCN1* encodes the regulatory subunit A of protein phosphatase 2A (PP2A-A) (Garbers *et al.*, 1996). The *pis1* (*polar auxin transport inhibitor-sensitive*) mutant is hypersensitive to NPA, and the wild-type gene was postulated to encode a protein that negatively regulates the function of exogenous auxin transport inhibitors (Fujita and Syono, 1997). Other mutants that show deviations in PAT are *pid* (*pinoid*) and *gn* (*gnom*). The *PID* gene encodes a protein-serine/threonine kinase that is induced by auxin and might function as a positive regulator of PAT (Benjamins *et al.*, 2001). GNOM functions in vesicle trafficking to the plasma membrane and is required for the polar localization

of PIN1 (Steinman *et al.*, 1999).

Regulation of the spatial and temporal distribution of auxin influx and efflux carriers provides the plant with an efficient mechanism to direct auxin flow to specific tissues and to precise groups of cells within these tissues. One can hypothesise that by changing the subcellular localisation of the carriers and their auxin transport capacity, the direction and rate of auxin flow can be modulated rapidly. The many newly discovered mutants with altered PAT, and the fact that some of the PAT proteins seem to be rapidly cycled between the plasma membrane and inner compartments of the cell (Geldner *et al.*, 2001) indicate that PAT is a tightly regulated yet very dynamic process.

Figure 1.
The pools of free and bound IAA are influenced by homeostatic mechanisms such as synthesis, compartmentation, catabolism and transport. The figure shows different mechanisms regulating input to and output from the pool of free IAA in a cell, together with some of the known components of IAA signal transduction pathways. Genes and gene products that are described and discussed in the text are included in the figure, and some of the regulatory mechanisms that affect biosynthesis, transport and signalling are indicated (broken lines).


IAA precursors
BUS1/SPS (CYP79F1)
RNT1/SUR2 (CYP83B1)
Indole glucosinolates
Compart-mentation
Trp-dependent and Trp-independent biosynthesis
SUR1
IAA
ILR1,IAR3
IAP1
IAA conjugates
Degradation products
Polar and phloem transport
AUX1
LAX1,2,3
PID,GN
PIS1
PIN1,2,3,4
TIR3 (BIG)
RCN1
Light
Phyto-chrome A
Perception
ABP1
Protein degradation (TIR1, AXR4)
ARF gene expression
Signal transduction
Early auxin-response genes: Aux/IAA (SHY2), SAURs, GH3-like, ACS, GH2/4-like
Membrane hyperpolarization, acidification of cell wall and apoplast
MAPKs, calcium/calmodulin
Late auxin-response genes: H+-ATPases, expansins, XET, EGase, K+ channels, cell cycle genes
Responses : Cell division, expansion and differentiation

Objectives of this study

Auxin plays a crucial role in plant growth and development. IAA homeostasis, i.e. the manner in which a plant regulates the concentration of IAA within specific cells through synthesis, metabolism and transport is not well understood. The work presented in this thesis addresses these issues, and the plant *Arabidopsis thaliana* (wall cress) was used for most of the studies. The use of *Arabidopsis* as a model organism has many advantages in plant physiology research (Meyerowitz and Sommerville, 1994; Meinke *et al.*, 1998), but the small size of the *Arabidopsis* seed is not one of them. Therefore, in our investigations of auxin homeostasis during germination and early seedling growth, analyses of seeds from *Pinus sylvestris* (Scots pine) were used to complement the *Arabidopsis* studies. This made it possible to get enough material for the planned experiments, and allowed the embryo and nutrient storage tissues in the seed to be analysed separately.

The following questions were addressed in the thesis:

- How do germinating seeds mobilise stored pools of IAA conjugates during the initial germination phase, and when do mechanisms of IAA metabolism (conjugation and catabolism) begin to be operational in the germinating seedling?

- When and where is IAA biosynthesis initiated in the germinating seedling and what pathways are involved?

- What is the spatial distribution of IAA within leaves and roots of different developmental stages, and how is this distribution correlated to developmental processes such as cell division, elongation and differentiation in these organs?

- Which tissues are the main sources of IAA for the developing seedling, and what impact do these sources have on the coordinated development of leaf and root tissues?

- How does transport of IAA in and between tissues affect IAA homeostasis and plant development, especially the development of primary and lateral roots?

Experimental

Plant material and growth conditions

The Columbia ecotype was used in all experiments performed with wild-type *Arabidopsis thaliana* (*At*) plants. Seeds were sterilised, put either on agar or on soil and cold treated for 3-4 days to synchronise germination and then grown either under long day (LD: 18 h light, 6 h darkness) or short day conditions (SD: 9 h light, 15 h darkness) at a temperature of 22-24 °C. Seeds, seedlings and expanding shoots from *Pinus sylvestris* were used in the investigations described in **Paper I**. In **Paper II**, analyses of expanding leaves from *Nicotiana tabacum* complemented the experiments performed on *Arabidopsis* plants. Two *Arabidopsis* auxin transport mutants were used in the studies: *aux1* (**Paper V**) and *AtPIN4* (**Paper VI**). The *aux1* mutant has previously been characterised (Bennett *et al.*, 1996), and carries a mutation in a gene encoding an auxin influx carrier protein. The *AtPIN4* gene belongs to the PIN family of auxin efflux carriers (Palme and Gälweiler, 1999). The *axr4aux1* double mutant was assessed in **Paper III**. *axr4* is an auxin resistant mutant, and the *AXR4* gene has been suggested to play a role in controlled protein degradation *via* the ubiquitin-proteasome pathway (Hobbie and Estelle, 1995; Gray and Estelle, 2000). In **Paper II**, the auxin-overproducing mutants *sur1* and *sur2* were used. Both exhibit increased auxin levels in the seedling stage, although the *sur2* mutant reverts to wild-type 12-15 DAG (Barlier *et al.*, 2000). The *SUR2* gene encodes a cytochrome P450 CYP83B1 protein that is probably involved in indole glucosinolate biosynthesis (Barlier *et al.*, 2000; Bak *et al.*, 2001). A role for *SUR1* in synthesis of IAA has been suggested (Seo *et al.*, 1998) and the *SUR1* gene has been predicted to encode an aminotransferase (Gopalraj *et al.*, 1996).

Anatomical studies

GUS reporter gene constructs were utilised in order to monitor cell division activity or IAA levels. Constructs with promoters for the cell cycle genes *CYC1* and *CDC2* fused to *uidA* were used (**Paper II**) to monitor cell division activity in *Nicotiana tabacum* leaves. *DR5::uidA* (**Papers III, IV** and **VI**) consists of the synthetic auxin response element DR5 fused to the *uidA* reporter gene (Ulmasov *et al.*, 1997) in *At* Columbia background, and was used to monitor endogenous IAA levels. Lateral root primordia were counted and classified according to Malamy and Benfey (1997) (**Papers III** and **IV**).

Quantification of IAA by mass spectrometry

Quantifications of endogenous IAA levels were performed by the method of Edlund *et al.* (1995) with minor modifications (**Papers I, II, III, V** and **VI**). This method, combining capillary gas chromatography (GC) with selected reaction monitoring mass spectrometry (SRM-MS), using a stable isotope labelled internal standard

($^{13}C_6$-IAA), is very sensitive and selective and makes it possible to analyse IAA concentrations in sub-milligram amounts of plant tissue. The relatively simple and fast purification method and the high throughput possible with GC-MS analysis enables a large number of samples (around 15/day) to be processed, which is important considering the large biological variation that can exist between replicate samples when very small amounts of plant tissue are analysed. This technique has been used to monitor IAA distribution in a variety of plant species such as *Arabidopsis* (**Papers II** and **III**), tobacco (Edlund *et al.*, 1995; Chen *et al.*, 2001b), hybrid aspen (Tuominen, 1997), maize (Philippar *et al.*, 1999) and pine (**Paper I**; Uggla, 1998), and also in different tissues within these plants such as embryos and seeds, different parts of germinating seedlings, leaves, hypocotyls, root tissue and the cambial region of stems. The sensitivity of the method is illustrated in **Figure 2**, showing a chromatogram of IAA extracted and purified from the most apical 1 mm section of *Arabidopsis* seedling roots. The tissue analysed was pooled from 50 seedlings, and weighed approximately 0.5 mg, but as can be observed in the chromatogram, the low detection limit would enable IAA to be detected in as little as 0.1 mg of plant tissue. **Figure 3** shows the redistribution of IAA in gravistimulated maize coleoptiles, demonstrating the use of this technique in investigations of auxin induced growth.

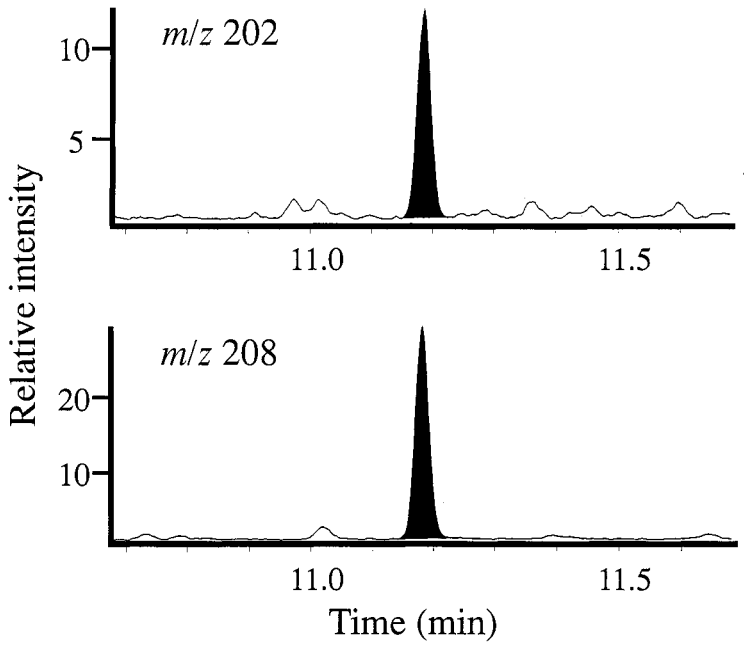
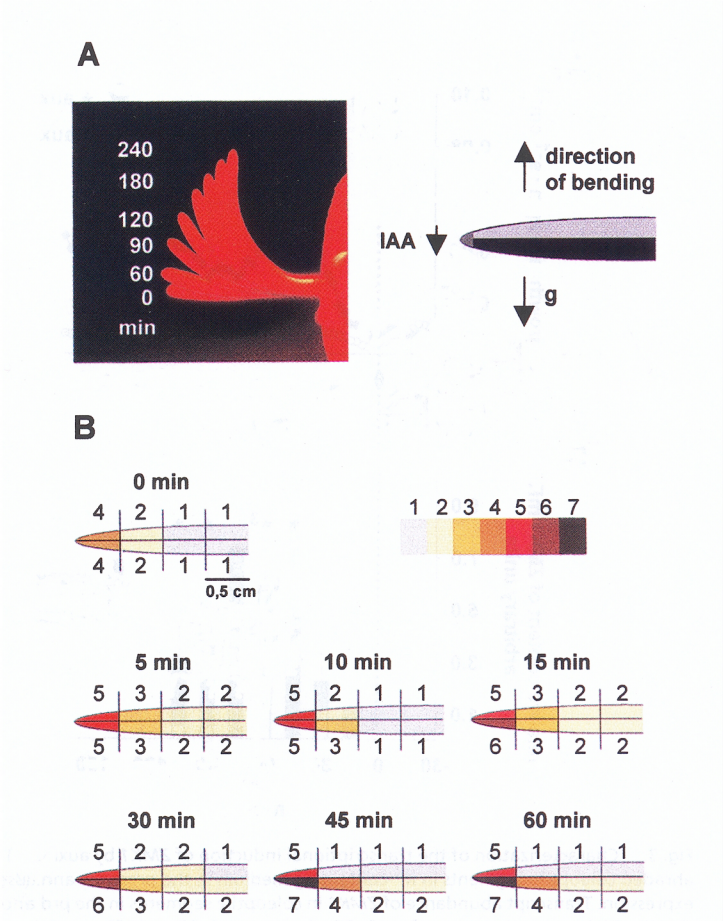


Figure 2.
Ion chromatogram of IAA (*m/z* 202) isolated from the most apical 1 mm section of *Arabidopsis* primary roots. Fifty 1 mm sections were pooled to get enough plant material for analysis (giving a total sample weight of approximately 0.5 mg), and 100 pg $^{13}C_6$-IAA (*m/z* 208) was added to the sample as an internal standard prior to extraction and purification. IAA was analysed as the IAA-Me-TMS derivative.



Coleoptile bending and redistribution of endogenous IAA concentration in response to gravistimulation. (*A Left*) Time-dependent coleoptile bending (0–240 min) in response to a 90° gravistimulation. (*Right*) Cartoon of a gravistimulated coleoptile. (*B*) IAA concentration (pg IAA/mg fresh weight, mean of $n = 3$ experiments) in 0.5-cm segments of coleoptile halves, gravistimulated for 0, 5, 10, 15, 30, 45, and 60 min. The IAA spectrum was decomposed into seven concentration ranges and highlighted by a color code. **1**, 10.8–13.9 ± 3.0; **2**, 14.1–17.9 ± 3.3; **3**, 19.9–23.5 ± 5.5; **4**, 26.9–31.2 ± 5.9; **5**, 34.6–42.5 ± 7.6; **6**, 48.7–49.4 ± 6.5; **7**, 51.1–58.8 ± 8.0 pg IAA/mg fresh weight.

Figure 3.
The figure is reprinted with permission from Philippar, K., Fuchs, I., Lüthen, H., Hoth, S., Bauer, C.S., Haga, K., Thiel, G., Ljung, K., Sandberg, G., Böttger, M., Becker, D. and Hedrich, R. (1999). Auxin-induced K^+ channel expression represents an essential step in coleoptile growth and gravitropism. *Proc. Natl. Acad. Sci. USA* 96, 12186-12191.

Radioactive and stable-isotope labelled substances as tools in studies of IAA metabolism

The use of labelled substances in studies of plant metabolism has greatly increased our knowledge of different metabolic pathways in plants. Both stable-isotope labelled substances and radioactive substances can be used, depending on the experiments performed. Stable–isotope labelled substrates are preferred when the products formed are analysed by mass spectrometry. With this analytical technique precursor-product relationships can be established and pool sizes of the exogenous substrate, its product and the corresponding endogenous substances can be monitored. Feedings with $^{15}N_1$-Trp and deuterium oxide (^2H_2O) were performed in order to study initiation of IAA biosynthesis *via* the Trp-dependent and Trp-independent pathways in germinating Scots pine seedlings (**Paper I**). Pulse-labelling (Normanly, 1997) with $^{13}C_6$-IAA (**Paper I**) and ^{12}C-IAA (**Paper VI**) was exploited to study turnover of IAA in Scots pine and *Arabidopsis*.

In order to identify unknown metabolites in specific metabolic pathways, radioactive substrates are valuable tools. Therefore, feeding experiments with ^{14}C-IAA were used to discover putative IAA metabolites in Scots pine by liquid chromatography - radioactive counting (LC-RC) metabolic profiling (**Paper I**). Isolated radioactive metabolites were then identified by LC-MS and LC-MS/MS, and unknown IAA metabolites were derivatised in order to increase the sensitivity in the MS-analysis and to obtain informative mass spectra and daughter ion spectra. A time study was also conducted (**Paper I**), comparing the disappearance of ^{14}C-labelled IAA with the appearance of identified IAA catabolites and conjugates over time.

Feeding experiments with deuterium oxide were undertaken in order to measure IAA biosynthetic rates in different *Arabidopsis* and Scots pine tissues during early seedling growth (**Papers I, II, III** and **IV**). The redundancy in the IAA biosynthetic pathways and the fact that the different steps in these pathways are poorly understood has made it difficult to measure IAA biosynthesis by methods such as feeding with labelled precursors or measuring transcript levels of enzymes in the different pathways. However, the general precursor deuterium oxide (^2H_2O) has proven to be very useful in metabolic studies (Mitra *et al.*, 1976) and has greatly increased our knowledge of the role and timing of IAA biosynthesis in different plant species (Cooney *et al.*, 1991; Bialek *et al.*, 1992; Michalczuk *et al.*, 1992; Jensen and Bandurski, 1996). Feeding studies with deuterium oxide allow total IAA biosynthesis to be measured within specific tissues of a plant, regardless of what pathways are operating in the tissues at the time of feeding. Furthermore, ^2H_2O is easily taken up by the plant and enters all cellular compartments (Mitra *et al.*, 1976; Pengelly and Bandurski, 1983). The physiological effects of deuterium oxide on plants are minor when feeding is done with less than 40 % ^2H_2O, even if some effects on different organisms have been noticed (Kushner *et al.*, 1998). For example, excised embryos from barley seeds germinated on medium containing 40 % ^2H_2O for 6 days showed 18 % growth inhibition compared to embryos germinated on medium without ^2H_2O, but were otherwise normal (Mitra *et al.*, 1976). To minimise the effects of ^2H_2O on general plant metabolism we used short incubation times (from 6 to 48 h) and media

containing only 30 % ^2H_2O in the studies described in this thesis. A new analytical method based on GC - selected reaction monitoring (SRM) - MS was also developed that enabled us to measure, for the first time, IAA biosynthesis rates in 2 mm sections of the root tip (**Paper IV**). The SRM method attained higher sensitivity as well as higher selectivity compared to GC - high resolution selected ion monitoring (HR-SIM) - MS at a resolution of 10000.

Results and discussion

Germination and early seedling growth

Mobilisation of stored auxin reserves

During the initial phase of germination, growth is completely dependent on stored reserves of lipids, proteins and carbohydrates. The plant hormones ABA, GAs and auxins are also stored in seeds for use in germination and early seedling growth. IAA may be stored as the free hormone, ester-linked conjugates (in which IAA is linked to sugars or *myo*-inositol) or amide-linked conjugates (in which IAA is linked to specific amino acids, peptides or proteins) (reviewed in Sembdner *et al.*, 1994; Normanly, 1997; Normanly and Bartel, 1999; Ljung *et al.*, 2002). The principal IAA forms used for storage in the seed (prior to the release of IAA at later stages of plant development) appear to differ among plant species. In some species the ester-linked conjugates predominate while in other species most IAA is bound as amide-linked conjugates.

We observed that in Scots pine the majority of the seed reserves of IAA are in the form of ester-linked conjugates, and only minor proportions occur as the free hormone or amide-linked conjugates (**Paper I**). During the first days of germination, the level of ester-linked conjugates rapidly decreased while the level of free IAA increased dramatically, indicating hydrolysis of the ester pool to be the main source of the pulse of free IAA avaliable to the developing seedling (**Paper I**, Figure 1c). The majority of the pools of both free and bound IAA were found in the embryo and not in the nutrient storage tissue of the seed: an arrangement providing the germinating seedling with virtually immediate access to the IAA needed for growth and diverse developmental processes.

In *Arabidopsis* seeds, free IAA and ester-linked conjugates constitute only <1% and 4% of the total pool of IAA, respectively. The rest of the pool (95%) consists of amide-linked conjugates, with IAA-amino acids accounting for 17% and IAA protein conjugates 78% of the total IAA pool (Park *et al.*, 2001). IAA protein conjugates have also been found in bean (*Phaseolus vulgaris*), and a study by Bialek and Cohen (1989) showed that the levels of these types of conjugates rapidly decrease as the seeds start to germinate, indicating that hydrolysis of IAA protein conjugates might provide an important source of free IAA for the germinating seedling.

Recently, a gene encoding an IAA-binding protein (*IAP1*) has been cloned from bean (Walz *et al.*, 2002), and antibodies raised against a 3.6 kD IAA-protein from bean were observed to cross react with proteins in the cotyledons and radicle from *Arabidopsis* seeds. It is very likely that IAA protein conjugates also exist in other species, and that they play major roles in controlling IAA homeostasis during germination and early seedling growth (and possibly also later in development).

De novo *synthesis of IAA*

We observed that in germinating Scots pine seedlings, Trp-dependent IAA biosynthesis is initiated around 4 days after germination (DAG) and Trp-independent synthesis is initiated later, around 7 DAG (**Paper I**). The differential induction of the two pathways is interesting, and it is possible that these pathways are either utilised in different tissues during early seedling growth or that they are operational in the same tissues but differently regulated. These experiments were done on a whole seedling basis, and dissection of the seedlings into separate tissues before analysis might in the future give some answers as to whether or not there is tissue specific expression of the IAA biosynthetic pathways in Scots pine seedlings. The differences in the timing of induction of the IAA synthesis pathways indicate that they are under developmental control and that *de novo* synthesis is initiated in the germinating seedling when the stored pools of IAA are used up and there is a need for IAA in elongation growth.

Very little is known about the initiation and tissue specific expression of the Trp-dependent and Trp-independent IAA biosynthetic pathways in *Arabidopsis* during germination and early seedling growth. However, there are data indicating that the Trp-dependent and Trp-independent pathways contribute equally to IAA biosynthesis in one-week-old *Arabidopsis* seedlings, but that one week later the Trp-independent pathway is predominant and less than 10 % of the IAA is synthesised from Trp (Normanly, 1997).

We demonstrated in experiments reported in **Paper II** that in young *Arabidopsis* seedlings (10 DAG), all parts of the seedling (cotyledons, young and expanding leaves and root tissue) are able to synthesise IAA after incubation with ^2H_2O. The seedlings were dissected into different tissues before incubation in order to distinguish between IAA synthesised in specific parts of the plant and IAA transported to the respective tissues. The highest IAA synthesis capacity was observed in the youngest leaves (leaves 3 and smaller), although cotyledons also showed relatively high synthesis capacities (**Paper II**, Figure 3). Roots and expanding leaves (leaves 1+2) showed lower but significant rates of IAA synthesis. For comparison with IAA synthesis rates derived for these dissected tissues, similar measurements were also performed on intact seedlings (**Paper II**, Figure 4). In intact *Arabidopsis* seedlings, IAA biosynthesis rates were lower in the youngest leaves (leaves 3 and smaller) but higher in the root compared to tissues excised from seedlings before incubation, indicating that transport of newly synthesised IAA from shoot to root tissues was taking place in the intact seedlings. **Figure 4** illustrates the relationships between

newly synthesised IAA and the total IAA pool in different parts of *Arabidopsis* seedlings after incubation of intact plants and dissected tissues with ^2H_2O for 24 h. Dissecting the seedlings into different tissues before incubation could cause other effects besides preventing transport of IAA, e.g. wounding due to dissection might disturb IAA biosynthesis. Dissection could also alter the amount of nutrients and assimilates transported to and from the tissues, thereby causing changes in IAA biosynthesis rates. Nevertheless, despite these potential complications, this approach can confirm the existence of specific IAA biosynthetic sites within the plant.

A set of experiments was also included in which intact seedlings were incubated with medium containing both naphthylphthalamic acid (NPA) and ^2H_2O. The phytotropin NPA is believed to block PAT by binding to a protein associated with the auxin efflux carrier (Morris, 2000). NPA treatment is generally believed to trap IAA in the tissue where it has been synthesised, increasing the IAA content in that tissue. Surprisingly this was not observed; instead NPA treatment lowered the observed IAA synthesis rates in expanding leaves and cotyledons, and in young leaves and roots no significant differences in IAA biosynthesis rates were observed compared to intact seedlings incubated without NPA (**Paper II**, Figure 4). One possible explanation for these results is that the NPA treatment causes a transient increase in IAA concentration in expanding leaves and cotyledons that induces feedback inhibition of IAA biosynthesis, thereby lowering IAA biosynthetic rates in these tissues.

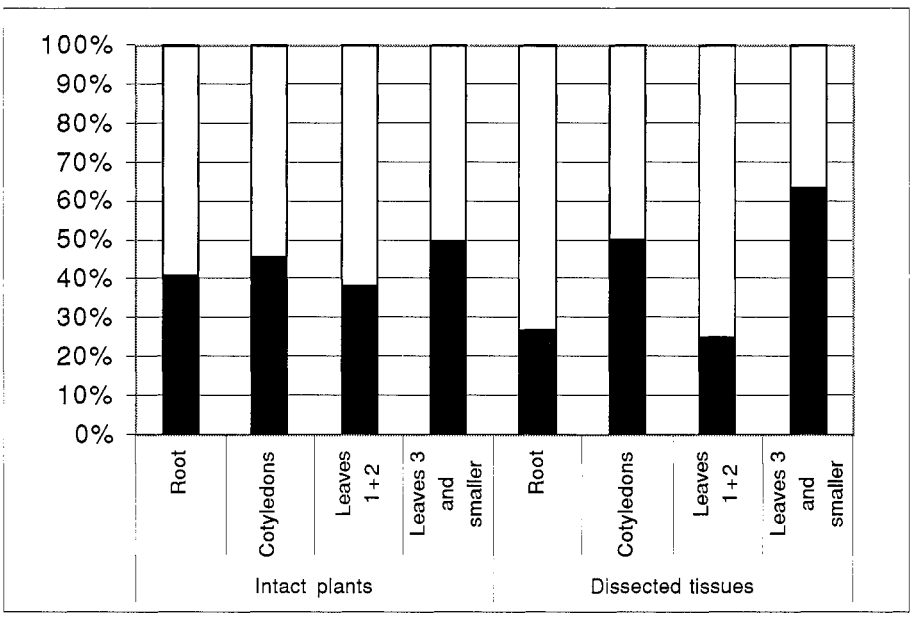


Figure 4.
Tissue specific de novo IAA biosynthesis was measured in Arabidopsis seedlings after incubation of intact plants or dissected tissues with medium containing 30 % deuterium oxide (^2H_2O) for 24 h. The black bars indicate the % of newly synthesised IAA compared to the total IAA pool (100 %).

This was further investigated in a time-course study with *Arabidopsis* seedlings that were incubated for 0-24 h with or without NPA in the medium, and the IAA concentration was then measured every second hour in both young and expanding leaves. The treatment with NPA caused a rapid increase in IAA levels in expanding leaves that peaked after 16 h, and then dropped again, supporting the existence of a feedback inhibition mechanism operating in this tissue (**Paper II**, Figure 5). In young leaves, NPA treatment did not cause any changes in IAA levels during this 24 h incubation period. Taken together, these results support the hypothesis that feedback inhibition of IAA biosynthesis is one mechanism by which the plant can regulate the size of the IAA pool in a specific tissue. Nothing is known about the molecular mechanisms involved in feedback inhibition of IAA synthesis, but this type of regulation has been observed in various other metabolic pathways, e.g. gibberellin biosynthesis (Yamaguchi and Kamiya, 2000). Direct measurements of the concentration of IAA conjugates and catabolites together with measurements of IAA concentration, biosynthetic rates and turnover, would increase our knowledge of the mechanisms controlling IAA homeostasis in different tissues during plant development and how they respond to perturbations in the IAA level.

Analyses of IAA biosynthesis rates and IAA levels in different plant tissues also made it possible to determine how different tissues contribute to the total pool of free IAA within the plant, and the turnover time of the pool in different organs. It was shown in our study (**Paper II**, Figure 6) that in 10-day-old *Arabidopsis* seedlings the root system and the expanding leaves (leaves 1+2) contained 38 % and 33 % of the total pool of IAA, respectively. Because of their small size, young leaves (leaves 3 and smaller, less than 4 mm in length) contained only 16 % of the total IAA pool despite their high IAA concentrations and high biosynthetic rates. The smallest IAA pool (13 %) was found in the cotyledons. The size of the tissue, the IAA concentration and biosynthesis rate as well as transport of the hormone to and from the tissue, are all factors that need to be considered when determining the importance of specific organs as sources and sinks of IAA.

IAA conjugation and catabolism

The major IAA conjugates and catabolic products were identified in Scots pine seedlings (**Paper I**). In addition to OxIAA and IAAsp, two novel conjugates were identified, namely IAAsp-*N*-glucoside and IAA-*N*-glucoside. IAA catabolism and conjugation commenced around 4 DAG with the formation of OxIAA and IAAsp, and around 6 DAG formation of IAAsp-*N*-glucoside and IAA-*N*-glucoside was observed. The timing of these events was correlated with the initiation of IAA biosynthesis, which was detected between 4 and 7 DAG. Pulse-labelling with $^{13}C_6$-IAA also indicated that there was rapid turnover of the IAA-pool 4-7 DAG. The timing of induction of IAA biosynthesis and catabolism was correlated to initiation of elongation growth in different tissues of the seedling such as the hypocotyl and root (4 DAG) and later the cotyledons (6 DAG) (**Paper I**, Figure 9). The results reported in **Paper I** collectively support the hypothesis that IAA homeostasis is under strict

developmental control in germinating Scots pine seedlings, and that conjugation and catabolism are important mechanisms regulating the IAA pool.

It was proposed by Östin *et al.* (1998) that IAGlu, IAAsp, OxIAA and OxIAA-hexose are major catabolic products of the IAA metabolic pathways in *Arabidopsis*. These metabolites were observed using metabolic profiling of plant extracts after feeding young seedlings with ^{14}C-IAA, and the putative IAA metabolites were then identified by GC-MS and LC-MS. In another study, the IAA conjugates IAAsp, IAGlu and IAGlc were identified in *Arabidopsis* (Tam *et al*, 2000). The concentration of these conjugates in young seedlings was found to be very low, and together they made up only 3 % of the total pool of IAA conjugates. IAGlc was found to represent 34 % of the pool of ester-linked conjugates and IAAsp and IAGlu together only 2 % of the amide-linked conjugate pool. The rest of the amide-linked conjugates are believed to consist of IAA conjugated to peptides and proteins, which are difficult to analyse by GC-MS (Park *et al.*, 2001; Walz *et al.*, 2002). In a general metabolic screen for indoles in *Arabidopsis*, two novel amide-linked IAA conjugates were recently identified, namely IAAla and IALeu, and the amounts of IAA and the metabolites OxIAA, IAAsp, IAGlu, IAAla and IALeu were quantified in specific tissues of *Arabidopsis* seedlings (Kowalczyk and Sandberg, 2001). It was observed that the youngest leaves and the root system contained the highest levels of IAAsp, IAGlu and OxIAA, as well as the highest levels of free IAA, indicating that these substances are irreversible catabolic products, formed in tissues with high rates of turnover of IAA. In contrast, the level of IALeu was high only in root tissue, whereas IAAla levels were high in all aerial tissues examined, and it was suggested that these are reversible conjugates that can be hydrolysed to yield free IAA. The discovery of genes encoding hydrolases that can release IAA from amino acid conjugates supports this observation, and so far two such genes, *ILR1* and *IAR3*, have been identified in *Arabidopsis* (Bartel and Fink, 1995; Davies *et al.*, 1999). The ILR1 enzyme has strong substrate specificity for IALeu and IAPhe, whereas the IAR3 enzyme displays the highest activity for IAAla.

The new methods developed to analyse endogenous levels of IAA catabolites and conjugates in *Arabidopsis* (Tam *et al.*, 2000; Kowalczyk and Sandberg, 2001) will be valuable tools in future investigations of the role of conjugation and catabolism in the regulation of IAA homeostasis. Further characterisation of the different IAA peptide and protein conjugates that are also present in *Arabidopsis* and the development of methods to quantify these substances are equally important. Earlier methods using alkaline hydrolysis of ester-linked and amide-linked conjugates to measure the total amount of bound IAA are imprecise, and the results obtained using them are also complicated by the fact that treatment of plant extracts with strong bases causes conversion of endogenous indole-3-acetonitrile (IAN), which is present in high concentrations in *Arabidopsis* tissues, to IAA (Ilic *et al.*, 1996). **Figure 5** presents a model for the developmental regulation of IAA homeostasis during germination and early seedling growth, showing important mechanisms controlling the IAA pool in the developing plant.

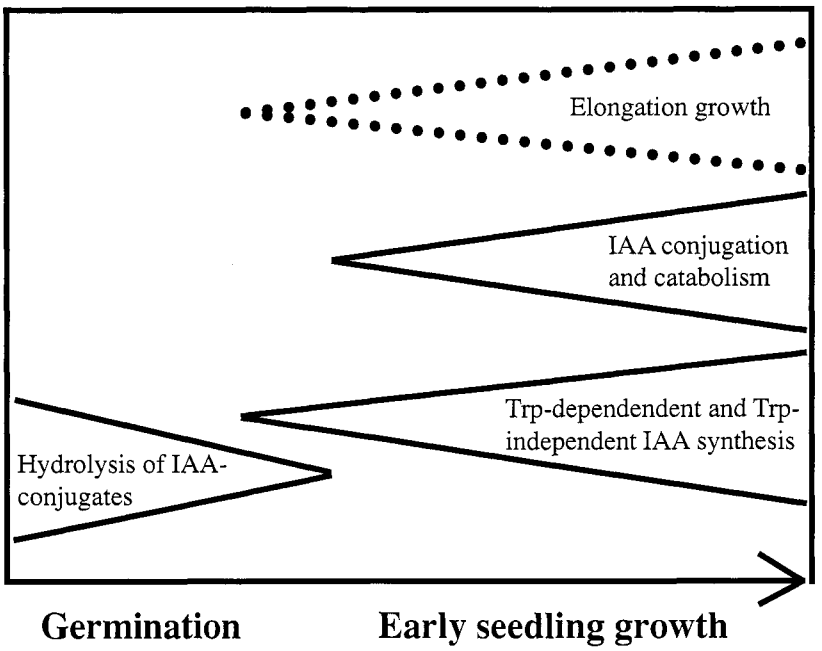


Figure 5.
A model describing different mechanisms regulating the pool of free IAA during germination and early seedling growth.

Leaf development

The shoot apical meristem

The formation of leaf primordia is initiated in the peripheral zone of the shoot apical meristem (SAM) in a pattern called phyllotaxis that is very regular and specific for each species. In *Arabidopsis* new leaf primordia are formed in a spiral pattern, giving rise to the rosette of leaves typical for this species (Medford *et al.*, 1992; Laufs *et al.*, 1998; Woodrick *et al.*, 2000). Growing evidence from investigations of mutants affected in SAM and leaf development indicate that cell-cell signalling between populations of cells within the shoot apex are crucial for SAM maintenance and organ formation (Fletcher and Meyerowitz, 2000; Clark, 2001; Haecker and Laux, 2001). The signals, which include various proteins, mRNA species, hormones and other small signalling molecules and ions, travel between neighbouring cells *via* receptors at the cell surface or through plasmodesmata. There is evidence for the existence of distinct symplastic fields within the SAM (Rinne and van der Schoot, 1998), and the position and gating of these connections between cells is proposed to be an important mechanism in controlling cell-to-cell signalling during development (Gisel *et al.*, 1999; van der Schoot and Rinne, 1999; Gisel *et al.*, 2002).

Different hypotheses have been postulated to explain the cellular mechanisms that result in the formation of new leaf primordia at the shoot apex. One theory presented by Green and co-workers suggests that biophysical mechanisms generate tension in specific parts of the shoot apex, resulting in buckling of the surface and, thus, formation of new leaf primordia (Selker *et al.*, 1992; Green 1994; Green, 1999). Other theories explain the formation of new leaf primordia by the existence of inhibitory fields created by existing primordia or gradients of specific morphogenic substances within the shoot apex (Holder, 1979; Lyndon, 1998). However, from the pioneering work by Snow and Snow in 1930-1940 onwards, auxin has been suggested to play an important role in phyllotaxis and leaf development (Snow and Snow, 1931, 1933, 1937). Recent research indicates that auxin transport and the formation of auxin gradients within the shoot apex are important for the positioning and development of leaf and flower primordia (Cleland, 2001; Kuhlemeier and Reinhardt, 2001). In the *Arabidopsis* mutant *pin1*, auxin transport is reduced by 90%, resulting in a pin-shaped inflorescence without normal flowers (Gälweiler *et al.*, 1998). The *pin1* phenotype can be mimicked by application of NPA (a PAT inhibitor) to the shoot apex, and it was proposed that *PIN1* regulates primordia separation, positioning and outgrowth as well as the expression of specific floral identity genes *via* local accumulation of auxin in the meristem (Vernoux *et al.*, 2000). Application of IAA to NPA-treated tomato meristems can induce the formation of leaf primordia, and on *Arabidopsis pin1* inflorescence apices it can induce the formation of flower primordia, supporting this hypothesis (Reinhardt *et al.*, 2000). New leaf primordia can also be induced by the local up-regulation of a cucumber expansin gene in transgenic tomato plants carrying an inducible promoter construct, by microinjection of anhydrotetracyclin (Ahtet) into the shoot apex (Pien *et al.*, 2001). Up-regulation of the expansin gene *LeExp18* in tomato was also observed at the site of initiation of new leaf primordia (Reinhardt *et al.*, 1998). Many of the known expansin genes are auxin inducible (Lee *et al.*, 2001), and a possible mechanism for leaf primordia formation could involve accumulation of auxin by PAT in specific groups of cells in the shoot apex, creating local auxin maxima and/or auxin gradients that lead to induction of specific expansin genes (as well as other downstream genes) at the site of primordium initiation. This process would induce cell division, cell expansion and cell differentiation, and finally lead to primordium outgrowth.

Leaf morphogenesis

Leaf morphogenesis involves the spatial and temporal regulation of cell division, cell expansion and cell differentiation in the developing leaf. These processes are believed to be co-ordinated by auxin and cytokinins, together with the expression of many different classes of genes, including homeobox genes and transcription factors, some induced by plant hormones. Many mutations affecting leaf development have been discovered and the function of these genes at the molecular level is now being investigated extensively (Dengler and Tsukaya, 2001; Pozzi *et al.*, 2001; Tasaka, 2001). However, in order to fully understand the effects these mutations exert on

leaf development, a basic understanding of plant morphology and anatomy is very important (Kaplan, 2001). Cell division is intense in the early stages of leaf development, giving rise to populations of cells that undergo differentiation as well as expansion growth. These processes have been investigated in *Arabidopsis* (Pyke *et al*, 1991; Van Lijsebettens and Clarke, 1998; Donnelly *et al.*, 1999) as well as other plant species, including both mono- and dicotyledons (Poethig and Sussex, 1985; Granier and Tardieu, 1998; Granier *et al.*, 2000; Tardieu and Granier, 2000). Environmental factors, such as light, temperature and water availability, can also act upon the plants' intrinsic developmental programmes to modify growth and development of leaves.

Although auxin is considered important for cell division, cell expansion and cell differentiation processes in plants; the cellular mechanisms that this hormone mediates during leaf development are just beginning to be understood. So, in order to improve our understanding of how auxin homeostasis could affect leaf development, a thorough investigation of endogenous IAA concentrations in *Arabidopsis* leaves at different developmental stages was undertaken (**Paper II**). We observed that the youngest developing leaves had the highest levels of the hormone, and that the IAA levels decreased almost a hundred-fold as the leaves expanded to their full size. In contrast, *Arabidopsis* cotyledons showed low and constant levels of IAA. Low, steady state levels of auxin were also found in older, fully expanded *Arabidopsis* leaves. In the leaves there were clear correlations between high IAA concentration and high rates of cell division, and this was observed in plants of different ages and in plants grown under different light conditions, giving generality to the negative correlation observed between IAA concentration and leaf size. These correlations observed in *Arabidopsis* were also verified by analyses of developing tobacco leaves (**Paper II**, Figure 2c). The larger size of the tobacco leaves made it possible to perform a high-resolution analysis of different tissues of the leaf throughout the leaf blade, which revealed high concentrations of IAA in actively dividing mesophyll tissue. In the parts of the tobacco leaf containing cells undergoing expansion growth, the IAA concentrations were much lower. We also investigated IAA synthesis in different *Arabidopsis* tissues and showed that the youngest developing leaves had the highest IAA biosynthetic rates of all tissues examined (**Paper II**, Figure 3). **Figure 6** describes the relationships observed between IAA concentration and leaf size in *Arabidopsis* plants during vegetative growth.

What conclusions can be drawn from these findings? Are the high IAA concentrations found in young developing leaves important for rapid cell division in these leaves, or do the dividing cells themselves produce high amounts of IAA, which may be needed for the differentiation of vascular tissue or other developmental processes within the plant? Both auxins and cytokinins stimulate cell division and cell differentiation in tissue culture of excised plant organs and isolated plant cells such as protoplasts (Krikorian, 1995). Furthermore, it is possible to manipulate organogenesis in tissue culture so that different tissue types (root, shoot or callus) are developed by changing the ratio between auxins and cytokinins added to the tissue culture medium. Nevertheless, the molecular basis of plant hormone action on cell division has been difficult to elucidate. In all eucaryotic cells, the progression through

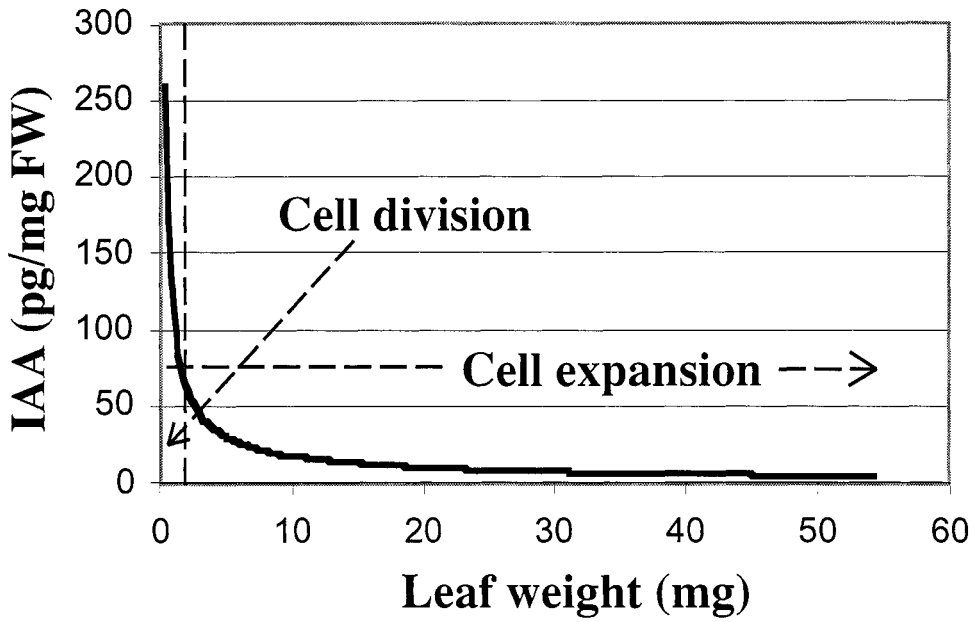


Figure 6.
A model of the relationship between leaf expansion and IAA concentration in developing Arabidopsis leaves.

the cell cycle is dependent on the expression and regulation of specific cyclin dependent kinases (CDKs), cyclins (cyc) and other regulatory proteins (Burssens *et al.*, 1998; Huntley and Murray, 1999; Mironov *et al.*, 1999). The cell cycle can be divided into several distinct phases: the S phase (DNA replication), the M phase (mitosis) and the G1 and G2 phases (intervals between the M/S and S/M phases). Cells can be arrested in different phases of the cell cycle, and both the G1-S and the G2-M transitions are important check points for cell cycle control. A role for auxin in regulation of the G1-S transition by activation of CDK-a has been suggested, based on data from *in vitro* experiments (Trehin *et al.*, 1998; Huntley and Murray, 1999; den Boer and Murray, 2000). However, these results need to be supported by *in vivo* experiments in order to get a better understanding of the role of auxin in cell cycle control during leaf development.

The importance of IAA for the development of vascular tissue in the leaf has been clearly demonstrated using PAT inhibitors (Mattsson *et al.*, 1999; Sieburth, 1999) as well as mutants with perturbed PAT or auxin responses (Berleth and Mattsson, 2000; Berleth *et al.*, 2000). Two different models have been proposed to explain the regulation of vascular development and vein patterning (Nelson and Dengler, 1997; Berleth, 2000). One model, called the 'diffusion-reaction prepattern hypothesis', suggests that autocatalysis and long-range inhibition of specific morphogenic substances could form a discrete pattern of vascular development, starting

from an initially uniform field. The other model is called the 'canalisation of signal flow hypothesis', suggesting that some cells are induced to become better auxin transporters than others. According to this hypothesis, the transport capacity of these cells increases with the flux of auxin, causing them to drain auxin from surrounding tissues and finally differentiate into vascular strands. These two models are not mutually exclusive but could in fact explain different aspects of vascular differentiation.

In order to investigate effects of altered auxin homeostasis on leaf development, we conducted a series of experiments in which we increased or decreased the IAA concentration in developing leaves and measured leaf expansion (**Paper II**). We used the auxin transport inhibitor NPA to block PAT in 10-day-old *Arabidopsis* seedlings, and effects on leaf expansion and IAA concentration in the leaves were measured after 1-5 days of this treatment (**Paper II**, Figure 7). As discussed earlier, this treatment induced feedback inhibition of IAA biosynthesis in expanding leaves from the NPA-treated seedlings, resulting in reduction of the IAA concentration in these leaves. A reduced rate of leaf expansion was also observed in the NPA treated seedlings. The effect of increased IAA levels on leaf expansion was investigated in the auxin overproducing mutants *sur1* and *sur2* during the first two weeks of seedling growth (**Paper II**, Figure 8). These mutants are perturbed in IAA and indole glucosinolate biosynthesis, respectively, and accumulate high levels of IAA in their tissues, especially early in development. The *sur2* mutant reverts to wild-type phenotype 12-15 DAG. The highest IAA levels were found in *sur1* leaves 1+2, and these leaves also showed the lowest leaf expansion rates (much lower than wild-type and lower than *sur2* leaves). Leaves 1+2 from *sur2* seedlings showed reduced leaf expansion rates, which correlated well with the higher IAA levels in these leaves, while leaves 3+4 showed normal leaf expansion and normal IAA levels. Taken together, these results indicate that normal leaf expansion is dependent on keeping the IAA concentration in the leaves at an optimal level for growth, as shown in **Figure 7**.

New findings regarding the function of PAT inhibitors suggest that their mode of action is more complex than earlier believed (Geldner *et al.*, 2001). All investigated PAT inhibitors (NPA, TIBA, PBA and HFCA) were found to block the actin dependent cycling of the PIN1 protein between the plasma membrane and some intracellular compartment in the cell, but they also inhibited vesicle-trafficking of proteins in general. Rapid cycling of PIN1 protein was found to be essential for the function of PIN1 in auxin transport, and the physiological effects observed after treatment with PAT inhibitors could be mimicked by treatment with BFA (brefeldin A), a substance known to inhibit vesicle-trafficking. The finding that PAT inhibitors have a more general inhibitory effect on the transport of membrane proteins is interesting, and raises questions about the specificity of these substances. Therefore, the possibility cannot be excluded that some of the physiological effects found in plants after treatment with PAT inhibitors like NPA are due to more general effects on protein transport, and perhaps also on plant growth and development.

A role for ABP1 in mediating leaf expansion was suggested by Jones *et al.* (1998) using inducible overexpression of *Arabidopsis ABP1* in leaves from transgenic

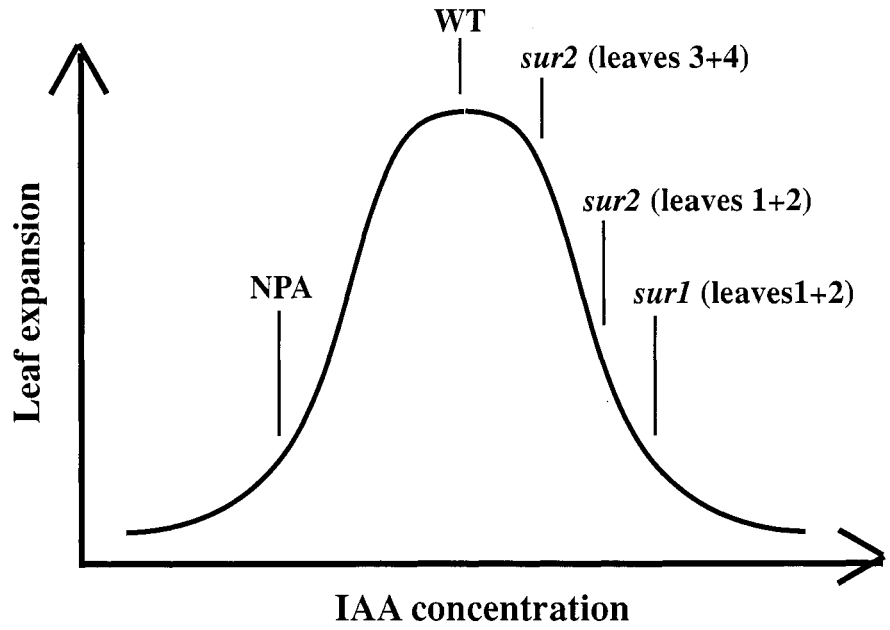


Figure 7.
Normal leaf expansion in Arabidopsis is dependent on maintaining an optimal IAA concentration in the developing leaves. Perturbation in IAA homeostasis within the leaf reduces leaf expansion.

tobacco plants. In their studies, strips of tobacco leaf tissue were incubated with solutions containing anhydrotetracyclin (to induce expression of *ABP1*) and 1-naphthaleneacetic acid (an auxin analogue), and auxin-induced growth was then measured from the curvature of the leaf strips. The treatment resulted in strong induction of cell expansion in strips coming from the basal part of the leaf, which is not normally responsive to auxin treatment. These results are supported by investigations of ABP1 distribution in tobacco leaves of different developmental stages, indicating a strong positive correlation between ABP1 abundance and cell expansion (Chen *et al.*, 2001b). Interestingly, the highest abundance of ABP1 was observed in the tip of the leaf (which was undergoing rapid cell expansion), and low amounts of ABP1 were found at the base of the leaf (which had the highest IAA levels and showed intense cell division). Auxin-induced growth is also dependent on the influx of K^+ ions into the cell via potassium channels located at the plasma membrane (Clausen *et al.*, 1997). In gravitropically stimulated maize coleoptiles, a specific gene (*ZmK1*) encoding a potassium channel protein was found to be induced after redistribution of auxin to the elongating lower half of the coleoptile (Philippar *et al.*, 1999; **Figure 3**). Genes encoding potassium channel proteins have also been identified in *Arabidopsis*, but nothing is known about the role of these proteins in leaf expansion, or if the corresponding genes can also be induced by auxin. It has been suggested that ABP1 can interact directly with ion channels at the

surface of the plasma membrane (Timpte, 2001), thereby transmitting the auxin signal to the interior of the cell.

Expansins and other cell wall modifying proteins like xyloglucan endotransglycosylase (XET) and endo-1,4-β-glucanase (EGase) have been found to be auxin-regulated in tissues of various plant species (Catalá *et al.*, 1997; Hutchison *et al.*, 1999; Caderas *et al.*, 2000; Catalá *et al.*, 2000; Catalá *et al.*, 2001). Analysis of the promoter region of expansin genes has shown the presence of auxin-responsive elements as well as responsive elements for other plant hormones (ABA, GAs and ethylene), especially in genes for α-expansins (Lee *et al.*, 2001). The redundancy in the number of genes encoding expansins (in *Arabidopsis* there are 26 α-expansins, five β-expansins and at least three expansin-like genes) indicate that they are involved in many different processes in plants that involve modification of cell walls. There are also indications that expansins are involved in leaf growth as well as in leaf morphogenesis (Cho and Cosgrove, 2000; Pien *et al.*, 2001).

There are strong indications that auxin influences many aspects of leaf morphogenesis, but the mechanisms involved are still poorly understood. A mature leaf contains many different cell types, and the many cellular processes involved in leaf growth and development clearly depend on the orchestration of a wide range of signal transduction pathways and the induction of numerous genes involved in these pathways. Key regulators in these processes are likely to involve auxin-inducible genes like the *Aux/IAA* genes. Investigations on the *Aux/IAA*-like genes *PttIAA1-8* from hybrid aspen have shown that they are strongly induced in young leaves by IAA treatment, whereas in mature leaves they are no longer inducible by auxin, indicating that they are under developmental control (Moyle *et al.*, 2002). As the leaves expand and mature, not only does the concentration of IAA decrease in the leaves, but it seems as if the leaves also lose their capacity to induce specific genes, thereby providing the plant with mechanisms to switch off specific signal transduction pathways.

Root development

Development of the primary root

The simple organisation of the *Arabidopsis* root makes it an ideal system for the study of plant morphogenesis. Mutants affected in root development, the use of laser ablation of root cells and cell-type-specific marker lines are beginning to unravel the basic principles of cell differentiation and organ formation in the root (Dolan *et al.*, 1993; Benfey and Scheres, 2000). It is now established that four types of initials (stem cells) located around the non-dividing cells of the quiescent centre (QC) give rise to all the different cell types in the developing root. The cells of the QC are believed to keep the initials in an undifferentiated state. The root and shoot meristems are formed in the embryo, and studies of mutants with perturbed PAT and

auxin responses, as well as embryos treated with synthetic auxins and PAT inhibitors, have shown that auxin transport and the creation of local auxin maxima in the embryo are essential for normal plant development (Costa and Dolan, 2000). The creation of polarised cells also seems to be an important factor for cell differentiation and morphogenesis in general (Grebe *et al.*, 2001). Treatment of roots with BFA has been shown to alter the distribution of IAA within the root apex and to inhibit AUX1 membrane trafficking, leading to changes in both cell polarity and root development (Grebe *et al.*, 2002). The effects of the vesicle trafficking inhibitor BFA and the mutant *gnom* on PIN1 cycling and polar localisation also indicate that PAT is needed to establish cell polarity in the developing embryo (Steinmann *et al.*, 1999; Geldner *et al.*, 2001).

The formation of a local auxin maximum in the *Arabidopsis* root tip was first detected using reporter constructs with synthetic auxin response elements (*DR5::uidA*) (Ulmasov *et al.*, 1997). Root tips of *DR5::uidA* transgenic seedlings showed maximum GUS activity in the columella initials (located distal to the QC) and lower levels of expression in the QC and the mature columella root cap. Furthermore, the expression and localisation of the GUS activity is altered in auxin response and PAT mutants, and also by the application of PAT inhibitors and auxin (Sabatini *et al.*, 1999). Changes in root patterning and polarity were also observed, indicating the importance of this local IAA maximum for normal root development.

To confirm the existence of a local IAA maximum in the root tip, we performed direct measurements of the IAA concentration in 1 mm sections of the root tip (**Paper III**, Figure 4). The outermost mm of the root tip showed the highest IAA level in root tips from 6 to 7-day-old seedlings. The gradient was formed between 3 and 6 DAG, and during that time an increase in DR5-GUS expression in the root tip was also observed (**Paper IV**). Measurements of IAA biosynthesis rates in different tissues of young *Arabidopsis* seedlings showed that root tissues have the capacity for *de novo* synthesis of IAA (**Papers II** and **III**), and experiments performed on dissected roots (**Paper II**) confirmed that this newly synthesised IAA was produced in the root system itself, and not transported there from aerial tissues. In order to pinpoint the site of IAA biosynthesis within the root, we developed a new, more sensitive analytical method that allowed measurements of synthesis rates in 2 mm sections of the root tip (**Paper IV**). Incubation experiments with medium containing 30 % deuterated water were performed both on intact plants and on roots from which the aerial parts of the plant had been removed before incubation, and the incorporation of deuterium into the IAA-molecule was determined by mass spectrometry isotopomer analysis. The highest IAA biosynthesis rates were observed in the most apical 2 mm section of the primary root from intact plants as well as from dissected roots (**Paper IV**, Figure 3). Since this method allows calculation of both IAA biosynthesis rate and IAA concentration in the same sample, it was possible to confirm the existence of a basipetal IAA gradient in the root tip from intact seedlings as early as 4 DAG (**Paper IV**, Figure 4). Interestingly, the IAA gradient was much weaker (8 DAG) or disappeared (4 DAG) in roots incubated without aerial parts, indicating the importance of NPA-independent transport of IAA from the shoot to the root for the formation of the basipetal root tip gradient. Removing the apical parts of

Arabidopsis seedlings has been shown to reduce DR5-GUS expression in the root tip and also to lower the endogenous IAA level in the root (Eklöf, 2001). The results of our direct IAA measurements and immunolocalisation studies suggest that the auxin influx and efflux carriers AUX1, AtPIN1 and AtPIN4 play important roles in mediating the formation of this gradient (**Papers V** and **VI**). Localisation of the AUX1 protein to protophloem cells of the root stele indicates a role for this protein in facilitating delivery of IAA to the root tip from the phloem (**Paper V**). Measurements of IAA concentration in the root tip of wild type and *aux1* seedlings supported this theory, showing that IAA accumulation in the root tip of *aux1* was disrupted compared to wild-type roots (**Paper V**, Figure 2a). AUX1 was also expressed in gravity-sensing columella cells and in the lateral root cap. The auxin efflux carrier AtPIN1 was found to be localised in cells in the vascular cylinder of the *Arabidopsis* root whereas the AtPIN4 protein was detected in the QC as well as the surrounding cells of the root meristem (**Paper VI**). **Figure 8a** illustrates the tissue-specific localisation of AUX1 and PIN4 protein in *Arabidopsis* roots. The cellular localisation of AtPIN4 indicates that the function of this protein is to generate an auxin maximum below the QC in the root apex. Taken together, these results suggest that the cells below the QC function as sinks for auxin transported down from more basal parts of the primary root, and that this sink is generated *via* AtPIN4 as well as other auxin efflux and influx carriers located in specific cell types of the root tip. **Figure 8b** shows a schematic diagram of how the basipetal auxin gradient found in the primary root tip is disrupted by treatment with PAT inhibitors and by removing the aerial parts of the plant.

Interestingly, the IAA gradient cannot be maintained solely by IAA biosynthesis in the root tip (**Paper IV**), and IAA coming from shoot tissues is needed for the formation of the gradient. If the auxin gradient found in the primary root tip is mainly generated by IAA derived from aerial tissues, what role is there for the newly discovered site of auxin biosynthesis in the root apex? We observed that aerially-derived IAA was needed for LRP emergence, but initiation of LRP was independent of IAA coming from the shoot (**Paper III**). Thus, this initiation phase is probably dependent on a local IAA source within the root tip (**Paper IV**). We have yet to identify precisely which cells in the root apex synthesise IAA, since the apical 2 mm of the root tip contains not only the root cap and the root meristem, but also the elongation zone and the differentiation zone where LR formation is initiated (Schiefelbein and Benfey, 1994). In order to pinpoint the IAA biosynthetic site to a more specific zone in the root apex, the sensitivity of the IAA biosynthesis measurements has to be increased even further. There are also many other aspects of root IAA biosynthesis and transport that need to be investigated further. For instance, do lateral roots have synthesis capacity in the same way as the primary root and, if so, when is this synthesis initiated? When is IAA biosynthesis initiated in the primary root during germination, and how is this synthesis regulated? Is root-synthesised IAA transported from the root apex to other tissues in the root, and, if so, how does this influence root development? Investigations of mutants with defects in different aspects of root development and IAA biosynthesis might help to answer some of these questions.

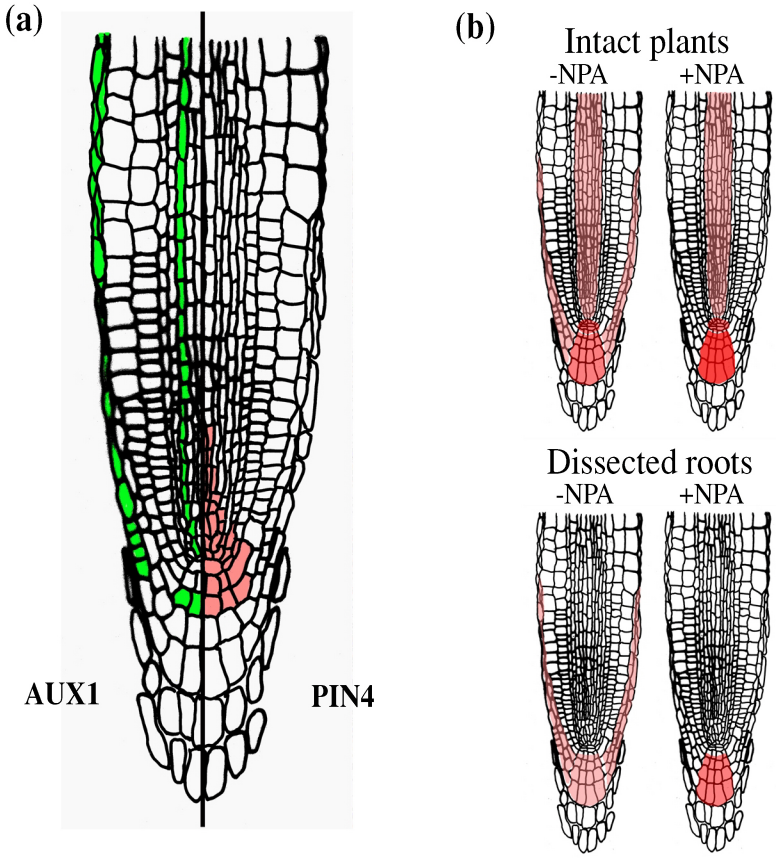


Figure 8.
a) Tissue-specific localisation of the auxin influx and efflux carriers AUX1 and PIN4 in Arabidopsis root tips. For a description of the different tissue types located in the Arabidopsis root apex see **Paper V**, Figure 1.
b) Different auxin transport mechanisms operate in the Arabidopsis primary root, resulting in the formation of a local auxin maximum within the root apex. If auxin transport is blocked either by PAT inhibitors or by removing aerial tissues, the auxin distribution changes within the root tip.

Lateral root development

The first lateral root primordium (LRP) in the *Arabidopsis* root is formed within 48 h of germination at a constant distance from the root apex (Beeckman *et al*., 2001). LRP are formed from pericycle cells in the differentiation zone of the root tip, but only from pericycle cells that are in contact with the xylem poles. These cells are arrested in the G1 phase of the cell cycle after entering the elongation zone, but become competent to divide and to initiate LRP when entering the differentiation zone of the root apex. Lateral roots are formed at a distance of 1.4 ± 0.28 mm from the root tip, based on the expression of cell-cycle markers indicating the initial stages of lateral root formation. Based on patterns of cell division and cell-type-specific marker lines, lateral root development has been divided into eight distinct stages

(stages I-VII and emergence) (Malamy and Benfey, 1997b).

The importance of auxin for the induction and emergence of lateral roots is well documented (Malamy and Benfey, 1997a; Casimiro *et al.*, 2001; Marchant *et al.*, 2002), but we are only just beginning to understand the mechanisms involved in these processes. A transcription activator called *NAC1* has been shown to act after *TIR1* (a gene involved in protein degradation *via* the ubiquitin pathway) promoting lateral root development (Xie *et al.*, 2000). This transcription factor activates two downstream auxin responsive genes called *DBP* (a lysine-rich DNA binding protein) and *AIR3* (a subtilisin-like protease). The mutated gene responsible for the phenotypic deviations in the newly described mutant *lin1* (*lateral root initiation 1*) (Malamy and Ryan, 2001) might have a role in co-ordinating lateral root initiation with environmental conditions like nutrient availability. Defects in other genes that have putative functions in LR initiation and development, like *alf3* and *alf4* (Celenza *et al.*, 1995) have been described, but these genes have not yet been cloned and characterised. The *Aux/IAA* gene *SHY2/IAA3* (Tian and Reed, 1999) has been suggested to act as both a positive and a negative regulator of auxin responses, in different situations, and mutations in it affect diverse aspects of root development. For instance, loss-of-function *shy2* seedlings showed increased lateral root formation compared to wild-type. Recently, a role for a Ran binding protein (AtRanBP1c) in root growth and lateral root initiation was suggested (Kim *et al.*, 2001). Ran binding proteins are active in nuclear transport of proteins and cell cycle progression, and it has been suggested that AtRanBP1c is involved in the delivery of proteins to the nucleus that might suppress auxin action and/or act in the regulation of the cell cycle.

The importance of PAT for root gravitropism and lateral root development has been clearly demonstrated in studies involving PAT mutants and the use of PAT inhibitors (Müller *et al.*, 1998; Marchant *et al.*, 1999; Marchant *et al.*, 2002; Casimiro *et al.*, 2001). For instance, treatment of roots with NPA caused redistribution of IAA in the root tip and an accumulation of IAA in the most apical 3 mm of the root in a study by Casimiro *et al.* (2001), and DR5-GUS expression was also up-regulated in the most apical 0.1 mm of the root apex in NPA-treated seedlings. Two different auxin transport pathways are believed to exist in the root, one providing acropetal (from the base of the root to the root tip) transport in the stele and the other basipetal (from the root tip towards the base of the root) transport in the epidermal and cortical cells of the root apex (Jones, 1998). It has been observed that basipetal transport of IAA in the root tip was essential for normal root gravitropism (Rashotte *et al.*, 2000) as well as lateral root initiation and root elongation (Rashotte *et al.*, 2000; Casimiro *et al.*, 2001). These events take place in a part of the root apex that is spatially separated from the IAA maximum in the most apical mm of the root tip (Casimiro *et al.*, 2001).

The auxin needed for LR development might originate from more than one source within the plant. PAT and/or phloem transport of IAA from aerial tissues could provide the root with auxin needed for growth and development. In parallel, IAA could also be synthesised in the root itself, e.g. in the root apex and the newly formed meristems in developing lateral roots. We analysed IAA content in different parts of

Arabidopsis seedlings during germination and early seedling growth and observed a transient increase of IAA concentration in the root system 5-7 DAG, at the same time as the emergence of the first lateral roots (**Paper III**, Figure 1). Experiments involving the excision of all aerial parts of the plants, or just the cotyledons, at different developmental stages showed that shoot-derived auxin (probably originating from the first developing leaves) was needed for lateral root emergence (**Paper III**, Figure 3). We also observed that application of IAA to the cut surface of dissected roots restored the frequency of LR emergence to wild-type levels and that removal of the aerial parts of the seedling reduced the IAA content in the root (**Paper III**; Eklöf, 2001). On the other hand, LRP initiation was not affected by removing IAA coming from the shoot (**Paper III**) and we also demonstrated that the primary root tip contained an autonomous IAA biosynthesis site (**Paper IV**). These observations all support the hypothesis that a source of auxin within the root tip is responsible for LRP initiation, whereas auxin coming from source tissues in the shoot is needed for the emergence of LRP (at least early in seedling development).

Coordination of plant growth and development

Sources and sinks of auxin

Young leaves and the shoot apex are believed to be the main sources of IAA for the plant. This is mainly based on the facts that apical tissues are rich in auxin, that PAT is directed towards the base of the plant in stem tissues, and that removal of apical parts of the plant or treatment with PAT inhibitors decreases the auxin content in the stem. However, in order to identify sources of a specific compound in plants definitively and unambiguously it is important not only to measure its endogenous concentration in different tissues, but equally important to measure biosynthesis rates, turnover and transport capacity to putative sinks. A high level of a specific substance in a tissue is not necessarily a reliable indicator that it is produced there. It may alternatively indicate that the tissue is a sink for the compound, generated by transport from other tissues.

Studies of IAA biosynthesis in *Arabidopsis* and other plants have usually been done on a whole plant basis, making it impossible to evaluate biosynthetic rates in specific tissues. We have developed new, very sensitive methods of analysis that now make it possible not only to analyse endogenous IAA concentrations, but also to measure *de novo* IAA biosynthesis in milligram amounts or less of plant tissue. These methods were used to identify putative sources of IAA in *Arabidopsis* seedlings (**Papers II, III** and **IV**). In our studies, young developing leaves were shown to comprise an important source of IAA based on the high endogenous IAA concentrations and high synthesis rates we observed in them (**Papers II** and **III**). Older leaves and cotyledons were also demonstrated to have some capacity for IAA synthesis, but at considerably lower rates (**Paper II**). Nevertheless, they might still represent significant IAA sources due to their relatively large mass and, probably,

higher IAA transport capacity (at least for true leaves since the vasculature in cotyledons is less well developed). The IAA produced in these source tissues is then transported *via* the phloem or PAT to sink tissues such as the root system (**Paper III**) where it profoundly affects root development. Experiments with dissected roots showed that the root system itself also has the capacity to synthesise auxin (**Paper II**). The discovery of a local source of auxin in the most apical 2 mm of the root tip (**Paper IV**) is a novel observation, and it is still only possible to speculate about the physiological relevance of this finding. It is tempting to suggest that this auxin source may play a role in lateral root initiation and/or root meristem development and maintenance. It will also be interesting to see if the cells that synthesise auxin are the same as those that display the local auxin maximum in the most apical mm of the root tip, or if the local auxin source is located in some other part of the root, e.g. in the root meristem or in the elongation and differentiation zones of the root tip.

Studies of NPA-treated vegetative meristems and the inflorescence meristems of PAT mutants indicate that the shoot meristem may also act as sinks for auxin (Reinhardt *et al.*, 2000). The source of this auxin would then probably be young leaves close to the apex that have gained IAA biosynthesis and transport capacity. After reaching the meristem, either transported in the phloem together with photoassimilates or *via* PAT, the cellular distribution of AtPIN1 and other influx and other efflux carriers could then direct the flow of auxin to form a local maximum, thus triggering the formation of new leaf primordia (Reinhardt *et al.*, 2000; Vernoux *et al.*, 2000; Cleland, 2001).

Auxin transport

There is growing evidence for the importance of both short-distance as well as long-distance transport of IAA in plant development (Lomax, 1995; Jones, 1998; Berleth and Sachs, 2001). Investigations on the tissue and cell specific localisation of the different influx and efflux carriers in *Arabidopsis* have greatly increased our knowledge of PAT and the roles that this directed flow of auxin might play in plant development (Morris, 2000; Swarup *et al.*, 2000; Muday and DeLong, 2001). Short range PAT appears to be of great significance in both root and shoot morphogenesis, and expression of specific PAT genes has been observed even in early phases of embryo development (Costa and Dolan, 2000). The cellular localisation of carriers provides the plant with a system for auxin canalisation that can direct the flow to specific parts of the developing embryo, or to the root or shoot apex, creating local auxin maxima. Long-range transport is mediated either by PAT in specialised cells associated with vascular tissues or *via* transport in the phloem. It is clear that PAT in the cambium is important for developmental processes such as cambial cell differentiation and xylogenesis in the stem (Mellerowicz, 2001). The auxin efflux carrier protein AtPIN1 was shown by Gälweiler *et al.* (1998) to be localised at the basal end of young and parenchymatous xylem cells and in cambial cells of inflorescence axes. However, very little is known about the temporal and spatial expression and cellular localisation of influx and efflux carriers in *Arabidopsis* leaves, petioles and stem tissues. Further investigations in this research area might answer some of the

questions regarding long-range IAA transport, and the relative importance of PAT versus phloem transport of IAA.

Expression of the influx carriers *AUX1* and *LAX2* in non-vascular and vascular tissues of young leaves indicate a function for these genes in the delivery of auxin to the phloem (Marchant *et al.*, 2002). AUX1 protein has also been found to be localised in specific cell files in the protophloem of the root apex (**Paper V**, Figure 1), suggesting that *AUX1* facilitates auxin unloading from the phloem into the root tip. Phloem transport of auxin provides the plant with a much faster auxin transport route than PAT, and this might be important for the plant in signalling between apical tissues and the root system. When leaves develop, they change from being sinks for assimilates and other substances coming from older, more developed leaves to being sources for these substances (Turgeon, 1989). The sink-source transition process in *Arabidopsis* leaves appears to be gradual, and strong expression of a phloem loading GUS-marker (*CmGAS1::uidA*) was found in cotyledon veins and in the majority of the veins in the oldest leaves (leaves 1+2) from a 14 DAG plant (Haritatos *et al.*, 2000). Developing leaves showed a basipetal staining pattern, with intense staining of small veins in the upper half of the leaf and progressively weaker staining towards the base of the leaf. The youngest leaves did not show any staining at all. It has not yet been demonstrated if the mechanism whereby IAA is loaded into the phloem is similar to the loading of assimilates, but the finding that developing leaves from *Arabidopsis* seedlings show active phloem loading is interesting. It also gives some support to the hypotheses that the first developing leaves contribute to the pulse of IAA reaching the root system observed 5-7 DAG (**Paper III**), and that this transport is mediated *via* the phloem. We found high levels of IAA in the petiole of developing *Arabidopsis* and tobacco leaves (**Paper II**, Figure 2), providing another indication that IAA is transported from leaves to other tissues. Closer examination of the expression patterns for the phloem loading marker CmGAS1-GUS and markers for the auxin influx carriers during the first week of germination might give more information about the development of transport capacity in the first true leaves and the cotyledons (and if this development is directly related to any increase in auxin transported to the root system). Both PAT as well as transport of IAA to and from tissues *via* the phloem has to be considered in future investigations on how auxin transport influences different aspects of plant development. **Figure 9** illustrates auxin transport in plants, directing the flow of auxin from putative sources to putative sink tissues.

Auxin gradients and morphogenesis

The concept of morphogens and morphogenic gradients or fields comes originally from animal research (Wolpert, 1996). Morphogens are believed to be synthesised in (or transported to) a specific part of the organism, where they set up a concentration gradient. The gradient then acts on cells in that region, providing them with positional information and thus affecting cell differentiation and subsequent morphogenesis (Day and Lawrence, 2000; Teleman *et al.*, 2001; Gurdon and Bourillot, 2001). The idea that plant hormones like auxins and cytokinins can influence

pattern formation has existed for many years (Holder, 1979; Steves and Sussex, 1989; Sachs, 1991). Plant hormones are small, easily diffusible substances that are active in very low concentrations in the cells. The unique PAT mechanism also makes auxin a likely candidate for a morphogen.

The first real evidence for the existence of auxin gradients in plant tissue came with the discovery of a sharp radial auxin concentration gradient in the vascular cambium of pine trees (Uggla *et al.*, 1996). The highest concentration of IAA was found in the cambial zone and its immediate derivatives, whereas the levels in the maturing phloem and xylem tissues were much lower. The width and amplitude of the gradient were found to influence the development of xylem cells, and it was postulated that auxin might function as a morphogen, providing positional information to the cells in the cambial region. The importance of PAT and the formation of the auxin gradient for cambial activity and wood formation is well supported, and new evidence points to an intricate interplay between auxin and other factors, such as sugars (that also form sharp concentration gradients), other plant hormones and transcription factors (Mellerowicz *et al.*, 2001; Uggla *et al.*, 2001; Newman and Campbell, 2000; Baima *et al.*, 2001).

In this thesis new evidence for the existence of at least two other types of auxin gradients in plants is presented. First, we observed a basipetal auxin concentration gradient in developing tobacco leaves (**Paper II**). Auxin levels were found to be very high in mesophyll tissue with high rates of cell division from the basal part of leaves, in contrast to areas at the tip and margin of the leaf, where only cell expansion took place and auxin concentrations were much lower. These findings are consistent with auxin involvement in leaf developmental processes such as cell division, cell expansion and the differentiation of vascular tissue. The other gradient was observed in *Arabidopsis* root tips (**Paper III**). Disruption of the auxin maximum in the root tip has been observed to cause defects in patterning and polarity of the root apex (Sabatini *et al.*, 1999). Based on this and other investigations on mutants related to auxin signalling and transport, a role for auxin as a morphogen in root development has been suggested (Doerner, 2000; Scheres, 2000). Recent research has also identified genes encoding transcription factors that appear to be involved in patterning of the root cortex, endodermis and epidermis (Benfey and Scheres, 2000; Costa and Dolan, 2000). The role of auxin in root patterning is largely unknown and much needs to be understood about the formation of the auxin gradient and auxin signalling in the root tip, as well as the interplay between auxin and other factors that affect morphogenesis.

These findings indicate that auxin gradients are formed in meristematic tissues and other tissues with high rates of cell division, and they support the hypothesis that auxin can function as a morphogen during plant growth and development. The challenge for future research lies in elucidating the signal transduction pathways that auxin acts upon and how they influence plant morphogenesis and organ formation. It is clear from the observations presented in this thesis that auxin gradients are more common in plants than previously thought, and that the auxin influx and efflux carriers play important roles in setting up these gradients in different plant tissues.

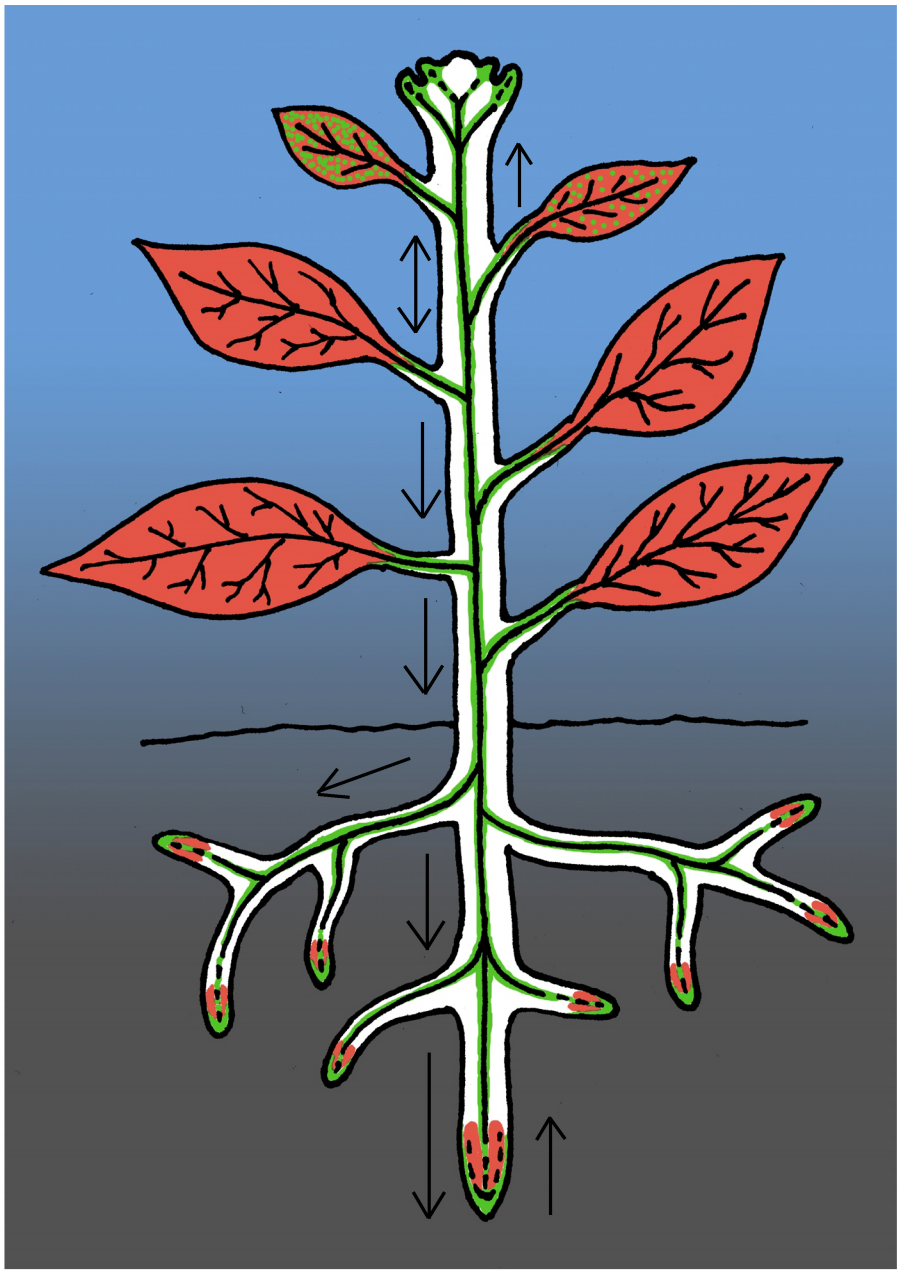


Figure 9.
A model of sources (red, IAA biosynthetic sites) and sinks (green, created by PAT and phloem transport of IAA) of auxin in plants, and putative transport routes for IAA synthesised *de novo* (arrows).

Conclusions and future prospects

The papers that this thesis is based upon deal with various features of auxin homeostasis, including biosynthesis, metabolism and transport. Auxin influences many aspects of plant development, some of which have been addressed in this thesis. The main goal has been to investigate the role of auxin in germination and early seedling growth and in the coordinated development of leaf and root tissues. Although this is a very broad area of research, interesting results have emerged that give increased insight into the role of auxin during plant development. Below is a summary of the findings that I find most interesting.

- Hydrolysis of ester-linked conjugates of IAA stored in the embryo provides germinating Scots pine seedlings with a pool of accessible free IAA.

- *De novo* IAA biosynthesis is temporally correlated with the induction of IAA catabolism in Scots pine seedlings, demonstrating the need for mechanisms to control IAA levels when IAA biosynthesis is initiated in the germinating seedling.

- In both *Arabidopsis* and tobacco, high IAA concentrations occur in leaves and leaf tissues with high rates of cell division and rapid development of vascular tissues. The lowest IAA levels were found in fully expanded leaves, and a strong negative correlation between leaf size and IAA concentration was observed.

- A tight regulation of IAA concentration appears to be a prerequisite for nomal leaf development in *Arabidopsis*. Feedback inhibition of IAA biosynthesis was observed after treating seedlings with NPA to block polar auxin transport.

- The highest rates of IAA biosynthesis were found in the youngest developing leaves of *Arabidopsis* seedlings, but cotyledons, older leaves and root tissue also showed *de novo* synthesis of IAA.

- The first developing leaves are probably the main source for the IAA pulse reaching the root system 5-7 DAG. This shoot derived IAA was essential to the emergence of lateral root primordia in *Arabidopsis* seedlings.

- A new method for IAA biosynthesis measurements was developed that enabled us to locate a source of IAA within the outermost 2 mm of the *Arabidopsis* root.

- A basipetal IAA gradient was observed in the *Arabidopsis* root tip, probably created by the cell specific localisation of the auxin influx and efflux carriers AUX1 and PIN4. Basipetal auxin transport from this local maximum was found to be important to root cell elongation and gravitropism.

Clearly, complex interactions between different plant tissues start early in germination and seedling growth, and are vitally important to plant development. There is a continuous need for short- as well as long-distance signalling in and between tissues to co-ordinate plant growth and developmental processes, and data presented in this thesis point to auxin being a major agent in this context. Many questions remain to be answered about the roles of the two auxin transport mechanisms that exist in plants (PAT and phloem transport) and their relative importance in different tissues, and during different stages of development. There are also many gaps in our knowledge concerning IAA biosynthesis, and a better understanding of the different pathways for IAA synthesis and their regulation is vital to a full understanding of IAA homeostasis. This also holds true for other aspects of IAA metabolism, such as conjugation and catabolism. Finally, the auxin signal transduction pathways that function in different tissues and during different developmental stages need to be further investigated in order to get a better understanding of the role of auxin in a developmental context.

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