Metabolism and Homeostasis of Indole-3-acetic Acid in *Arabidopsis thaliana*

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Abstract

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The endogenous concentration of a primary auxin, indole-3-acetic acid (IAA) is regulated by a number of biochemical processes, including transport, biosynthesis, catabolism and conjugation. The studies on which this thesis is based focused on an instrumental approach for investigating pathways of IAA metabolism and for examining the IAA biochemistry in various *Arabidopsis thaliana* mutants.

We have identified the major low molecular weight IAA metabolites in *Arabidopsis* with isotope labelling studies using exogenous IAA at different concentrations. The endogenous presence of these metabolites was subsequently confirmed and their internal concentrations were measured using highly sensitive liquid chromatography-mass spectrometry (LC-MS) techniques. The results showed that non-decarboxylative oxidation of IAA is a key catabolic pathway in *A. thaliana*. The levels of selected ester and amide conjugates were very low, indicating either a rapid turnover or a low metabolite flux through the main conjugation pathways. Although the biosynthetic details of IAA amide conjugates remain unknown, an enzyme involved in ester conjugate biosynthesis was cloned and characterised at the biochemical level.

The developed methods were subsequently used to analyse two mutants with suspected defects in auxin homeostasis, *bus* and *sur2*. In both cases we demonstrated that although IAA metabolism was profoundly altered, the cause of these alterations was not related to the hormone's conjugation or catabolism. Instead, these mutations distorted the secondary metabolism of specific amino acids (methionine and tryptophan, respectively), which indirectly affected IAA biosynthesis.

We also developed analytical tools to investigate proteins involved in IAA metabolism. In conjunction with standard protein analysis techniques, these methods can be used to identify enzymatic activities involved in IAA biosynthesis pathways, either dependent or independent of tryptophan. In relation to this, a second LC-MS technique for the *in vivo* determination of IAA precursors is also presented.

Keywords: auxin, conjugation, catabolism, biosynthesis, liquid chromatography, mass spectrometry, multiple reaction monitoring

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List of publications:

I. Östin, A., **Kowalczyk, M.**, Bhalerao, R. P. and Sandberg, G. (1998). Metabolism of indole-3-acetic acid in *Arabidopsis*. *Plant Physiology*, **118**, 285-296.

II. Barlier, I.*, **Kowalczyk, M.***, Marchant, A.*, Ljung, K., Bhalerao, R., Bennett, M., Sandberg, G. and Bellini, C. (2000). The *SUR2* gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 14819-14824.

III. Reintanz, B., Lehnen, M., Reichelt, M., Gershenzon, J., **Kowalczyk, M.**, Sandberg, G., Godde, M., Uhl, R. and Palme, K. (2001). *bus*, a bushy *Arabidopsis* CYP79F1 knockout mutant with abolished synthesis of short-chain aliphatic glucosinolates. *Plant Cell*, **13**, 351-367.

IV. Jackson, R. G., Lim, E. K., Li, Y., **Kowalczyk, M.**, Sandberg, G., Hoggett, J., Ashford, D. A. and Bowles, D. J. (2001). Identification and biochemical characterization of an *Arabidopsis* indole-3-acetic acid glucosyltransferase. *Journal of Biological Chemistry*, **276**, 4350-4356.

V. Dolušić, E., **Kowalczyk, M.**, Magnus, V., Sandberg, G. and Normanly, J. (2001). Biotinylated indoles as probes for indole-binding proteins. *Bioconjugate Chemistry*, **12**, 152-162.

VI. **Kowalczyk, M.** and Sandberg, G. 2001. Quantitative analysis of indole-3-acetic acid metabolites in *Arabidopsis*. *Plant Physiology*, **127**, 1845-1853.

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Publications are referred to in the text by the respective Roman numerals.

Introduction

Background

Auxins are a class of phytohormones that naturally occur in all plants. They are implicated in virtually all aspects of plant growth and development. The concept of a substance that is able to regulate growth in localised plant parts was initially suggested from work on gravitropism and phototropism in the late XIX century (Ciesielski, 1872; Darwin & Darwin, 1880). The structure of the substance involved was later (in 1934) elucidated as an indole derivative – indole-3-acetic acid (IAA) (Haagen-Smit, 1951). Since then, additional natural auxins have been identified: indole-3-butyric acid (IBA; reviewed in Bartel *et al.*, 2001), 4-chloroindole-3-acetic acid (Cl-IAA; see Reinecke *et al.*, 1996, for more details) and phenylacetic acid (PA), which are less commonly found. Synthetic auxins with increased stability were also developed for agricultural use, *e.g.* 2,4-dichlorophenoxyacetic acid (2,4-D), naphtalene-1-acetic acid (NAA) and chiral auxin, indole-3-succinic acid (ISA; Armstrong *et al.*, 2002).

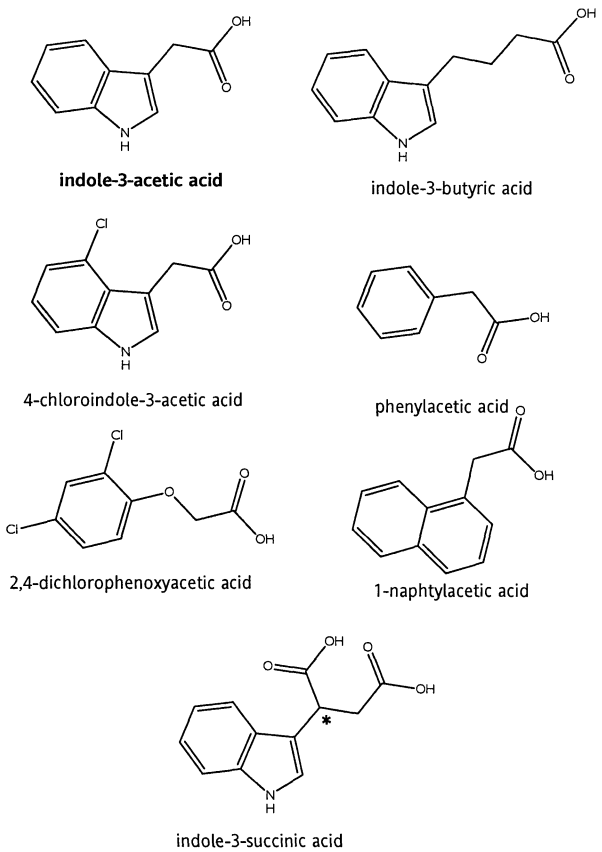

Figure 1: Natural and synthetic auxins.

Following the discovery of the first plant growth regulator, several other types of compounds that affect plant growth and development were identified. Based on their chemical properties, this group of growth regulators was divided into five classes: auxins, cytokinins, gibberellins, ethylene and abscisic acid. Nowadays this classification has mostly historical meaning, as further "hormonal" substances that do not fit into any of these classes but are able to influence, sometimes very specifically, various processes in plants have been discovered. Examples of such compounds include small molecules like brassinosteroids (Brosa, 1999), jasmonic acid (Creelman & Mullet, 1997), salicylic acid (McDowell & Dangl, 2000) and polyamines (Ghosh, 2000), in addition to larger peptides, like systemin (Gehring, 1999; Lindsey *et al.*, 2002). Clearly, the group of compounds referred to as plant hormones, phytohormones or plant growth regulators is very diverse with respect to their chemistry, the processes they regulate and their mode of action. To encompass this diversity, currently accepted definitions of plant hormones have to be somewhat ambiguous. According to these definitions, plant hormones are supposed to be relatively small organic molecules that, at very low concentrations, regulate physiological processes in plants. However, the similarity between animal hormones and plant hormones virtually ends with the name "hormone" (although there are chemical similarities in some cases). Unlike their animal counterparts, phytohormones are known to affect not only tissues and organs distant from their site of biosynthesis (autonomous mode of action), but also the site itself. In fact, this effect is in many cases essential for their action. Another important property, inferred from their structural and physiological variability, is that different classes of phytohormones have to act either in conjunction or in opposition to each other. The net result of these interactions is a developmental programme that offers amazing adaptability to changing environmental conditions and often ensures survival of the plant despite such changes. Indeed, in order to control these interactions, both the response to and the levels of the plant hormones have to be regulated. At the cellular level, signal transduction, transport and metabolism are the key processes providing the essential regulatory framework. Key aspects of auxin signalling and metabolism will be discussed in the following sections of this introduction, with particular emphasis on the model plant *Arabidopsis thaliana.* This choice of topic is of course personal, and auxin transport is certainly very important for plant physiology and development. Nevertheless, this subject has been quite extensively reviewed in the past two years (Friml & Palme, 2002; Muday, 2001; Muday & Murphy, 2002; Parry *et al.*, 2001) and a recent doctoral thesis from this department has also discussed it in detail (Ljung, 2002).

Auxin as a ligand

Recognition and binding of the auxin molecule is essential for all processes mentioned above. Since the discovery of auxin structure, numerous studies have attempted to identify (and explain) features of the molecule that are critical to its binding, and thus its activity. The first explanation of auxin activity, the charge separation model, was based on measurements of electrical charges on the atoms of IAA, and its synthetic analogue, 2,4-D. The model asserted that, for an organic molecule to act as an auxin, the local charge centres (a net positive charge, usually

associated with the ring system, and a negative charge located at the carboxyl group) have to be spaced at a distance of around 5 Å (for more details and references, see the review by Thimann, 1963). Accordingly, the binding site has to be constructed in such way that it allows accommodation of both charge centres, by means of appropriately spaced groups of opposite charge. However, subsequent experiments have proven that charge separation alone cannot account for the activity of auxin analogues, and furthermore that there was no correlation between the magnitude of the positive charge on the ring system and their activity (Farrimond *et al.*, 1981).

Advances in computational chemistry and the availability of precise IAA molecule measurements allowed the development of more advanced binding site models. For instance, Tomić and co-workers applied molecular dynamics simulations and modern molecular mechanics to construct a pharmacophoric model for the classification of various auxins and auxin-like substances using biological activity data (Tomic *et al.*, 1998). Based on their interaction properties, 50 organic compounds were divided into four classes with a range of auxin activities: strongly active, weakly active with a weak anti-auxin activity, inactive and inhibitory. All the compounds were then modelled in two conformations: planar ("P") and tilted ("T"). This concept originated from the observation that although the indole ring is a planar structure, the side chain has some freedom of movement and the energy barrier between the two conformations (in which the side chain is either coplanar with the ring or tilted out of the ring plane) is quite low. The results of the classification were subjected to similarity analysis, which showed that the "T" conformation approach produced better class differentiation. The calculations of surface properties for each ligand were then translated into a binding site map. Based on these results, it was proposed that the binding site should have a stable and planar electrophilic platform to accommodate the ring system and the adjacent carboxylic group acceptor. The acceptor should be separated from the platform by a hydrophobic transition zone to facilitate "T"-conformation ligand binding and/or possible conformational changes. Related studies of Edgerton and co-workers, although entirely based on binding characteristics of IAA and two isomers of NAA to the maize ABP1 protein, reached essentially the same conclusions (Edgerton *et al.*, 1994). Due to the relatively small size of the ligand, IAA binding sites in different proteins are likely to share similar features. Furthermore, some of these features are also very likely to be shared with other, unrelated proteins that use small, hydrophobic molecules with a polar side chain as substrates. This is most probably the case with glutathione S-transferase (Bilang & Sturm, 1995) and protein disulphide isomerase (Primm & Gilbert, 2001), both of which have low affinity binding sites for IAA.

Auxin signal transduction

Auxin perception in plants is still a matter of considerable debate. The strongest candidate for an auxin receptor is the ABP1 protein, but the hypothesis that it acts in this way has been proposed and rejected several times. Although another putative receptor site has recently been postulated (Chen, 2001), it most likely

constitutes a low affinity binding site, active only in high concentrations of the hormone. On the other hand, copious evidence, both direct and indirect, indicates that ABP1 may represent a high affinity receptor site. Regardless of the techniques used for identification, ABP1 has been found in numerous plant species, essentially because of its ability to bind auxin with a physiologically relevant affinity (for a review, see Napier, 1995). The vast majority of ABP1 molecules in the cell have been found inside the endoplasmic reticulum (ER), which is consistent with the fact that the protein contains a functional, C-terminal located ER retention sequence (called a KDEL or HDEL motif; Napier, 1997). However, a small fraction of the ABP1 molecules appear to be localized at the plasma membrane (Leblanc *et al.*, 1999).

Despite this apparent discrepancy between its potential function and cellular localization, experimental evidence supports the role of ABP1 as an auxin receptor, or at least an auxin response mediator. Controlled overexpression of the *Arabidopsis ABP1* gene in tobacco leaf strips resulted in increased capacity for auxin mediated cell expansion and, in the case of intact plants, a significant increase in the cell size(Jones *et al.*, 1998). The same results were also obtained in the homologous system, *i.e.* with the maize *ABP1* gene overexpressed in maize (Jones *et al.*, 1998). An inducible tobacco system was then used to investigate further the changes caused by overexpression of *ABP1* at the cellular level. It was established that overexpression induced nuclei in young tobacco leaf cells to advance prematurely to the G2 phase. This was considered an indirect effect of cell expansion, which occurred before the G2 advance. The highest level of ABP1 was correlated with maximal cell expansion, maximal auxin-inducible growth and the lowest free auxin levels, whereas maximal cell division was correlated with the highest auxin levels and the lowest ABP1 protein levels. When transcription of *ABP1* was suppressed by antisense transformation in the wild type tobacco cells, it caused strong reduction of cell size, with almost no effect on cell division (Chen, 2001). The cellular effects of *ABP1* overexpression were also tested in tobacco leaf guard cells (Bauly *et al.*, 2000), where exogenous auxin is known to affect inward and outward rectifying potassium channels. The cited authors found that, in comparison to wild type, auxin sensitivity of these channels was much higher in the *ABP1* overexpressors. Although different variants of the ER retention sequence in the protein were used (including non-functional ones), enrichment of the plasma membrane with ABP1 was not detected. The *ABP1* null mutant phenotype was also recently analysed (Chen *et al.*, 2001). An insertion in the *ABP1* gene was found to be lethal in the homozygotes, which died in the early globular phase of embryogenesis, indicating that ABP1 protein is essential for both viability and normal plant development.

Despite extensive research in this field, the auxin signal transduction system downstream of the putative receptor remains unclear. Analysis of *A. thaliana* auxin response mutants has led to a model in which auxin signalling is mediated by regulated protein degradation (reviewed in Leyser, 2001). According to this model, auxin sensitivity depends on the function of an SCF-type protein ubiquitin-ligase complex. This complex is formed from the SKP1 protein, the Cullin/CDC53 protein homologue, the "F-box" protein and the RBX1/ROC1 protein. The RBX1/ROC1 protein catalyses the formation of ubiquitin polymers, an activity

that requires Cullin/CDC53 to be present in the complex. The latter protein also functions as a regulatory unit for the SCF complex, since it can affect its nuclear localization and, by conjugation with members of the NEDD8/RUB1 protein family, regulate its enzymatic activity. The SKP1 protein interacts with both Cullin/CDC53 and the "F-box" protein and acts as a scaffold on which the complex is assembled. The "F-box" proteins are responsible for selection of the substrates, which are then ubiquitinated by the SCF complex and thus marked for degradation on the proteasome.

A number of *Arabidopsis* mutants that have reduced sensitivity to auxin and are defective in various aspects of the SCF complex function have been identified. Of particular interest in this group are *axr1*, bearing a mutation in the NEDD8/RUB1 protein (del Pozo *et al.*, 2002; del Pozo & Estelle, 1999) and *tir1*, in which one of the "F-box" proteins is mutated (Gray *et al.*, 1999). The target protein of the SCF complex has not been definitively determined, although Aux/IAA proteins are considered very likely candidates. Genes encoding Aux/IAA proteins, which are unique to the plant kingdom, were first identified because of the rapid induction of their expression by auxin. In *Arabidopsis*, 29 such genes have been found (Reed, 2001). Nine gain-of-function mutations in these genes cause defects in auxin responses and auxin regulated gene expression (Abel *et al.*, 1995b; Leyser *et al.*, 1996; Reed, 2001; Rogg *et al.*, 2001). Mutations from this group, predominantly localised in the conserved domain II of the polypeptide, seem to increase stability of the Aux/IAA proteins, suggesting that their turnover may be important for auxin signal transduction. Some of these mutants also display abnormal light-related phenotypes, including short hypocotyls in either light or dark conditions, as well as the ability to develop leaves in the dark. Further links between the auxin and light responses is indicated by the ability of phytochrome A to phosphorylate Aux/IAA proteins *in vitro* (Colon-Carmona *et al.*, 2000) (for a review on the interactions between auxin and light signalling pathways see Tian & Reed, 2001).

The Aux/IAA proteins regulate gene transcription via protein-protein interactions with a class of transcription regulators called ARF (auxin response factor) proteins. Binding to ARFs alters their activity, and this in turn affects gene expression. Three mutations in ARFs, *ettin* (Sessions *et al.*, 1997), *monopteros* (Hardtke & Berleth, 1998) and *msg1/nph4* (Stowe-Evans *et al.*, 1998) result in reduced auxin sensitivity as well as defects in tropic responses. These observations strongly indicate that ARFs mediate auxin signals. The ARF proteins bind to so-called "auxin response elements" in the promoter region of many auxin-regulated genes, including the *GH3* gene family, which is also known to be rapidly induced by the hormone and suspected to be involved in auxin signal mediation (Liu *et al.*, 1994; Ulmasov *et al.*, 1995; Ulmasov *et al.*, 1997).

Evidently, signal transmission has to occur between the nodes we are currently aware of: the putative receptor ABP1, the SCF complex and auxin regulated gene expression. Current knowledge of signal transduction cascades in both plant and animal systems suggests that protein phosphorylation and/or dephosporylation events may be implicated here. Kinase and phosphorylase activities regulated or induced by auxins are relatively well documented, and are known to influence a variety of cellular processes (Bogre *et al.*, 2000; Jonak *et al.*, 1994; Kovtun *et al.*,

1998; Mockaitis & Howell, 2000). Moreover, auxin signalling and transport pathways seem to be directly affected by some of these enzymes. For example, loss of PINOID kinase activity leads to severe alterations in auxin responses (Christensen *et al.*, 2000). Conversely, mutation in the regulatory subunit of the heterotrimeric protein phosphatase 2A results in altered growth responses and defects in auxin transport (Deruere *et al.*, 1999; Garbers *et al.*, 1996; Jackson & Soll, 1999). It should be pointed out that in some systems, phosphorylation of the protein often marks it for rapid degradation, mediated by the ubiquitin system (reviewed in Craig & Tyers, 1999; Karin & Ben-Neriah, 2000; Kornitzer & Ciechanover, 2000; Willems *et al.*, 1999). This evidence directly links protein phosphorylation to auxin signal transduction and obviously also to auxin-regulated gene expression. In this context, the previously mentioned phosphorylation of Aux/IAA proteins by phytochrome A is even more interesting.

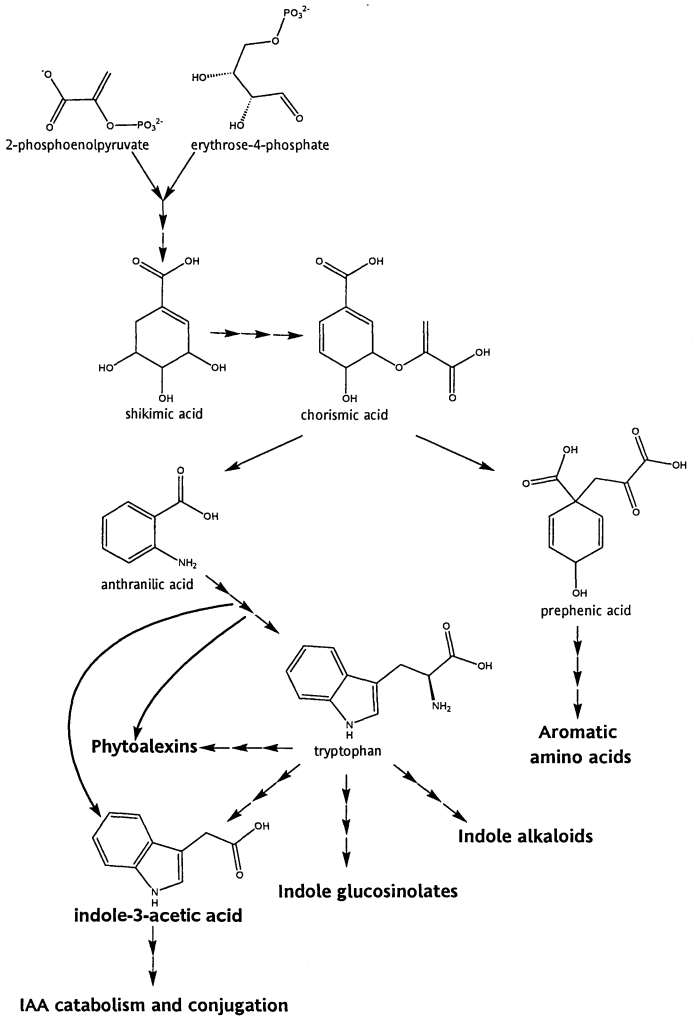

Figure 2: Biochemical context of the biosynthesis of tryptophan, IAA and other indole-derived compounds in plants.

Auxin metabolism

Biosynthesis and potential IAA precursors

Tryptophan-dependent biosynthesis

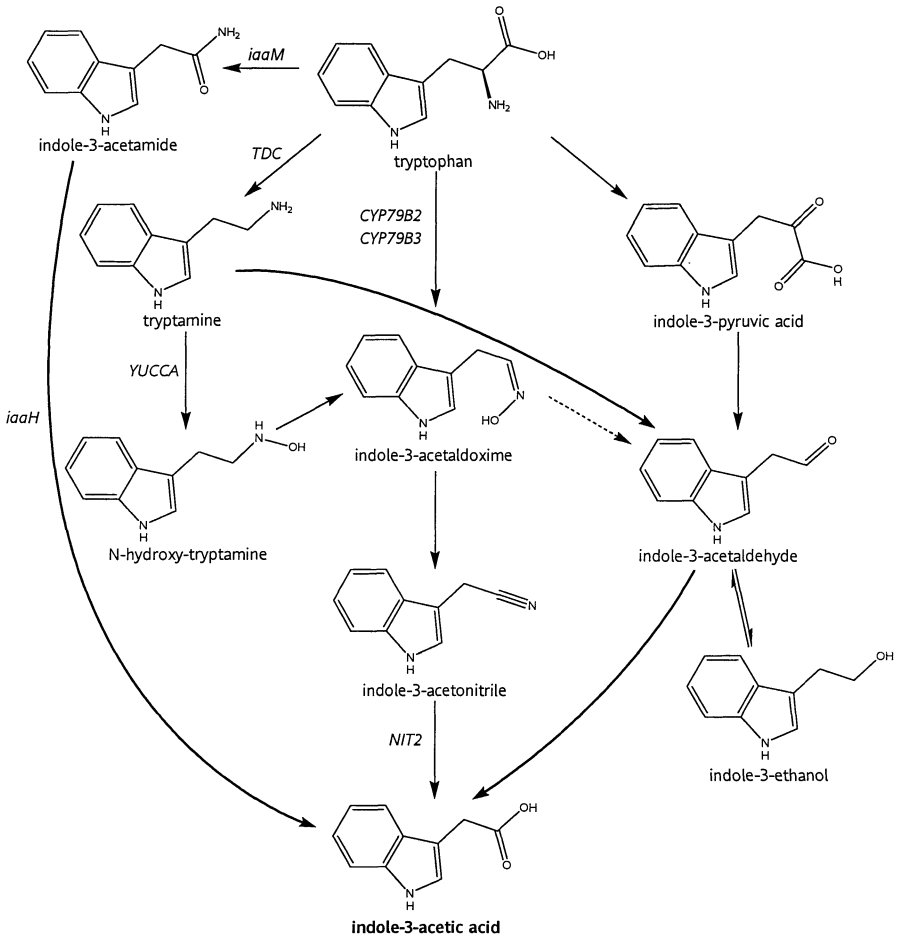

Figure 3: Proposed tryptophan-dependent pathways of IAA biosynthesis in plants and bacteria.

Although the biological activity of IAA is exclusive to plants, the ability to produce it is much more common. Numerous species of microorganisms, including pathogenic and epiphytic bacteria as well as pathogenic fungi (Costacurta & Vanderleyden, 1995; Glickmann *et al.*, 1998; Patten & Glick, 1996), can synthesize IAA and related compounds, like for example indole-3-lactic acid (ILA; Sprunck *et al.*, 1995) and indole-3-ethanol (IAEt; Rey *et al.*, 2001). These pathways may involve an array of intermediates, however the precursor for IAA is generally tryptophan. Perhaps the most common biosynthetic route involves indole-3-acetamide (IAM) as an intermediate, produced from tryptophan in the reaction catalysed by a flavoprotein, tryptophan 2-monooxygenase (Emanuele *et al.*, 1995) and then hydrolysed to IAA by IAM

hydrolase. The IAM pathway is often introduced into plants to overproduce auxin (see Sitbon *et al.*, 1992 for an example, and Kawaguchi & Syono, 1996 for a review on auxin overproduction from bacterial pathways), however it is not considered a native IAA biosynthesis route in plants. Instead, a number of other tryptophan-dependent IAA biosynthesis pathways have been postulated (recently reviewed in Bartel *et al.*, 2001 and Ljung *et al.*, 2002) and three major intermediates have been proposed: indole-3-pyruvic acid (IPyA), tryptamine and indole-3-acetonitrile (IAN).

The synthesis of IAA *via* IPyA is also known from various microorganisms (Brandl & Lindow, 1996; Koga *et al.*, 1994; Sergeeva *et al.*, 2002; Steenhoudt & Vanderleyden, 2000), and experimental data provide extensive support for its native occurrence in plants. The intermediate itself has been identified as an endogenous constituent in tomato shoots (Cooney & Nonhebel, 1989) and *Arabidopsis* seedlings (Tam & Normanly, 1998). Aminotransferase activity, possibly engaged in IPyA synthesis from Trp, has been reported in maize (Koshiba *et al.*, 1993), mung bean (Simpson *et al.*, 1997) and tobacco (Elbahr *et al.*, 1987) and its involvement in auxin overproduction in the *Arabidopsis sur1* mutant has been suggested (Gopalraj *et al.*, 1996). *In vivo* labelling studies with deuterium oxide have indicated that IPyA is a precursor to IAA in tomato shoots (Cooney & Nonhebel, 1991), however feedings with labelled synthetic compound were not performed in these investigations, due to its very unstable character. Synthesis of IAA from IPyA was proposed to occur *via* decarboxylation to indole-3-acetaldehyde (IAAld) and subsequent oxidation. Enzymatic activity of IPyA decarboxylase has not been reported in plants so far, although in *Arabidopsis* several aldehyde oxidase activities have been described and implicated in IAA biosynthesis (Sekimoto *et al.*, 1998; Seo *et al.*, 1998). Nevertheless, it is possible that instead of two sequential reactions, one oxidative decarboxylation reaction may occur without the release of any intermediate.

Another probable IAA biosynthetic pathway in plants involves decarboxylation of Trp to yield tryptamine, followed by an amino-to-carbonyl group exchange, thus producing IAAld, which is then oxidised to IAA. Endogenous tryptamine has been found in a range of plant species, including *Catharanthus roseus* (Tikhomiroff & Jolicoeur, 2002) and tomato (Cooney & Nonhebel, 1991). Its presence typically correlates well with reports of pyridoxal-5'-phosphate-dependent tryptophan decarboxylase (TDC) activity in the plants (Facchini *et al.*, 2000; Lopez-Meyer & Nessler, 1997). However, these plants usually produce indole alkaloids, for which tryptamine is a known precursor (Facchini, 2001). The involvement of tryptamine in IAA biosynthesis has been tested in tomato shoots using *in vivo* labelling with D_2O (Cooney & Nonhebel, 1991). It was found that the initial rates of deuterium incorporation were similar for IAA and tryptamine, and the total incorporation values did not exclude tryptamine as a precursor for IAA. However, with a longer incubation time tryptamine continued to accumulate label, while the labelling in IAA decreased, possibly due to precursor pool dilution with unlabelled tryptophan recycled from protein hydrolysis in slowly senescing tissue. This led to the conclusion that tryptamine is not an endogenous precursor of IAA in tomato shoots, and that tryptamine and IAA were apparently synthesized from different pools of tryptophan. The presence of such restricted

pools had been previously confirmed by comparison of the deuterium incorporation into IAA from D_2O and tryptophan. Further evidence that tryptamine is not likely to be a precursor for IAA comes from the results of *TDC*-overexpression in plants (Facchini *et al.*, 2000; Guillet *et al.*, 2000). No visible auxin-related phenotypes were observed, but overproduction of tryptamine caused a variety of other effects, including reduced levels of indole glucosinolates (Chavadej *et al.*, 1994), changes in shikimate metabolism and altered biosynthesis of aromatic amino acids (Guillet *et al.*, 2000).

Although neither tryptamine nor enzymatic activity of tryptophan decarboxylase has been found in *Arabidopsis*, interest in the tryptamine-mediated pathway has been renewed by the recent finding that *YUCCA*-overexpressing plants contain elevated levels of IAA. The *YUCCA* gene encodes a putative flavin monooxygenase, shown *in vitro* to catalyse N-hydroxylation of tryptamine (Zhao *et al.*, 2001). The product of this reaction is supposedly converted to indole-3-acetaldoxime (IAOx) and further to IAA (Bartel *et al.*, 2001; Zhao *et al.*, 2001). Not surprisingly, the *Arabidopsis* genome contains at least nine other *YUCCA*-like genes, and two of them have been shown to cause increases in IAA levels when overexpressed. An orthologue of *YUCCA* has recently been cloned from petunia, and its ectopic expression results in increased auxin levels (Tobena-Santamaria *et al.*, 2002).

The third proposed biosynthetic pathway involves indole-3-acetonitrile (IAN) as a direct intermediate to IAA. Apart from its putative role in auxin biosynthesis, indole-3-acetonitrile is also implicated in plant defence mechanisms and it has been shown to have strong antifungal activity (Pedras *et al.*, 2002). Production of IAN in *Brassica junicea* is stress- and pathogen- inducible (Pedras *et al.*, 2002), whereas in other *Brassica* species and *Arabidopsis* it is considered constitutive. Although myrosinase-catalysed breakdown of indole glucosinolates can also produce IAN, *in vivo* labelling studies have confirmed that the major IAN biosynthetic route occurs through indole-3-acetaldoxime. In elicited *B. junicea*, levels of deuterium incorporation from D_4-IAOx into IAN were shown to reach 24% after four days of incubation. In agreement with the proposed topology of this biosynthetic pathway, levels of incorporation from D_5-Trp and D_4-indole into IAN were 25% and 7%, respectively. Furthermore, two P450-dependent enzymes (CYP79B2 and CYP79B3) from *Arabidopsis* that are able to convert Trp to IAOx have been identified and cloned (Hull *et al.*, 2000; Mikkelsen *et al.*, 2000). Overexpression studies in bacteria, yeasts and plants fully established the function of these enzymes. In overexpressing plants, an increase in indole glucosinolate levels was also observed (Mikkelsen *et al.*, 2000). This finding is consistent with the anticipated role of IAOx as a precursor to indole glucosinolates. Interestingly, CYP79B2 mRNA accumulates in response to wounding and pathogen infection, indicating a possible connection between this gene and the plant defence system (Hull *et al.*, 2000; Mikkelsen *et al.*, 2000).

Compared to IAOx biosynthesis, its dehydration to IAN has been less thoroughly investigated. Although enzymatic activity catalysing such a reaction has been observed in Chinese cabbage (Ludwig-Muller & Hilgenberg, 1990), the only *Arabidopsis* enzyme known to use IAOx as a substrate, P450-dependent

CYP83B1, is unquestionably involved in biosynthesis of indole glucosinolates (Paper II, Bak *et al.*, 2001; Hansen *et al.*, 2001). However, loss-of-function mutation (*sur2*, allelic to *rnt1*) in the gene encoding this enzyme leads to a range of auxin overproduction phenotypes (Delarue *et al.*, 1998) in addition to significant reductions in indole glucosinolate contents (Bak *et al.*, 2001). This raises the possibility that unmetabolised excess IAOx may be converted to IAA through the IAN pathway. The results of *in vivo* D_5-Trp labelling studies with this mutant are, unfortunately, quite inconclusive in the context of this conversion (Paper II). On the other hand, introduction of D_4-IAOx into UV-irradiated leaves and roots of *B. rapa* resulted in 98% incorporation of D_4 into a number of metabolites, including IAEt, IAN, N-hydroxylated 2,3-diOxIAN and phytoalexins (Pedras *et al.*, 2002). Accumulation of IAAld was observed in certain stages of development of *sur2* plants and it is very likely to be a product of IAOx metabolism that could be directly converted to IAEt and possibly to IAA (Paper II). In fact, a biosynthetic pathway involving both IAOx and IAAld had also been proposed before this report, in Chinese cabbage (Helmlinger *et al.*, 1987). Nevertheless, the non-enzymatic conversion pathway from IAOx into IAAld *via* the imine intermediate (Paper II) is purely speculative, although it was rather uncritically accepted as part of the general IAA biosynthesis route in plants (Eckardt, 2001).

The final conversion of IAN to IAA is allegedly catalysed by a nitrilase. Activity of this enzyme has been found in various members of the *Cruciferae* and several other plant families (Bartling *et al.*, 1992; Thimann & Mahadevan, 1964). Based on its sequence homology to the *Arabidopsis NIT4* gene, a putative nitrilase has also been found in tobacco (Dohmoto *et al.*, 1999; Dohmoto *et al.*, 2000b). The *Arabidopsis* genome contains four genes encoding nitrilases (*NIT1* to *NIT4*; Bartel & Fink, 1994; Bartling *et al.*, 1992) and their ability to convert IAN into IAA has been confirmed *in vitro* (Vorwerk *et al.*, 2001). The IAN-mediated pathway has been proposed to act as a primary IAA biosynthetic route in *Arabidopsis* (Bartling *et al.*, 1994; Muller *et al.*, 1998) and an enzymatic complex of "IAA synthase" that includes nitrilase activity has been identified (Muller & Weiler, 2000). However, several lines of experimental evidence suggest that IAN may not participate in IAA biosynthesis to the expected level. The first three genes encoding nitrilases (NIT1 to NIT3) are located in tandem on the third *Arabidopsis* chromosome and share around 80% identity with each other. Their affinity for IAN and the velocity of the reaction with IAN appears to be lower than for other putative substrates tested (Vorwerk *et al.*, 2001). The fastest *in vitro* kinetic parameters were observed for conversion of phenylpropionitrile, allylcyanide, phenylthioacetonitrile and methylthioacetonitrile. Some of these compounds are natural products of the degradation of aliphatic and aromatic glucosinolates and may be native substrates for nitrilase. Interestingly, however, IAN conversion by a NIT2 enzyme displayed a rather unusual temperature optimum at 15°C and assaying its activity at this temperature resulted in significant improvements in its kinetic parameters (Vorwerk *et al.*, 2001). Overexpression of *NIT2* in *Arabidopsis* caused increased sensitivity to IAN, but it did not result in a typical auxin overproduction phenotype (Normanly *et al.*, 1997). Similar results were obtained from overexpression of *NIT2* in tobacco (Schmidt *et al.*, 1996), where again it

caused increased sensitivity to IAN (wild-type tobacco is not sensitive to IAN), but an auxin overproduction phenotype developed only after the application of exogenous IAN. Overexpression of *NIT1* and *NIT3* in tobacco caused relatively low exogenous IAN sensitivity (Dohmoto *et al.*, 2000a). Antisense *NIT1* and *NIT2 Arabidopsis* lines were reported to have reduced "total IAA" (a collective measure of free and conjugated IAA) levels, but the levels of free IAA were similar to those in the wild-type (Grsic *et al.*, 1998). Overexpression of the same genes in their normal orientation again did not cause any change in free hormone levels, although "total IAA" was increased. Collectively, these results provide evidence that NIT2, and possibly NIT1, enzymes take part in auxin metabolism, although details of their involvement are not very clear. The last nitrilase, NIT4 is less similar to the other three, and its gene is located on the fifth chromosome. This enzyme was recently proposed to operate in a cyanide detoxification pathway and is probably not involved in IAA biosynthesis (Piotrowski *et al.*, 2001). The NIT4 orthologue in tobacco may, presumably, have the same function.

Tryptophan-independent biosynthesis

The hypothesis that auxin can be synthesised in plants by a tryptophan independent pathway is derived from several independent lines of experimental evidence, although detailed biochemical organization of this pathway is still unknown. The most important evidence in favour of this hypothesis comes from stable isotope labelling studies applied to the analysis of tryptophan auxotrophic mutants. The maize *orp* (*orange pericarp*) mutant, which lacks activity of the Trp-synthase beta subunit (TSB), has been shown to have significantly increased levels of "total IAA". Labelling with D_2O confirmed that *de novo* synthesis of IAA takes place in *orp*, but no labelling of IAA from D_5-Trp was detected in these studies (Wright *et al.*, 1991). Similar experiments were performed with the *Arabidopsis trp2-1* mutant, a conditional tryptophan auxotroph also lacking TSB activity, but only in high light conditions (Last *et al.*, 1991). In *trp2-1*, the levels of "total IAA" are again increased, while the free hormone remains at wild-type levels. Double labelling studies with ^{15}N-anthranilate and D_5-Trp have shown much higher incorporation of ^{15}N label into IAA (around 40%) than into Trp (around 10%), and very little incorporation of D_5 label into IAA (also near 10%) (Normanly *et al.*, 1993). The D_5 value was claimed to be only slightly higher than values that would be generated by non-enzymatic conversion of Trp to IAA, but it is unclear how such conversion could actually occur.

Analysis of mutations in enzymes of the Trp biosynthesis pathway upstream of the Trp-synthase complex could provide crucial evidence for the presence of the Trp-independent pathway in plants, but regrettably little of the required information has been gathered to date. In the *trp1-1* mutant of *Arabidopsis*, which has a defective phosphoribosyl-anthranilate transferase (Last & Fink, 1988; Rose *et al.*, 1997), wild-type levels of both free hormone and "total IAA" have been reported (Normanly *et al.*, 1993), but details were never published. In contrast to the results obtained with tryptophan synthase mutants, reduction of the "total IAA" level was observed in transgenic *Arabidopsis* overexpressing indole-3-glycerol phosphate synthase in antisense orientation (Ouyang *et al.*, 2000). These plants also had decreased levels of soluble tryptophan, although not to the extent

observed in *trp2-1* or *trp3-1*. Unfortunately, no data on the free IAA levels are available in the cited publication.

Various aspects of auxin metabolism, including IAA biosynthesis, have been extensively investigated in maize endosperm. This system is believed to synthesize IAA mostly through the Trp-dependent pathway (Rekoslavskaya, 1995): a hypothesis that has been confirmed by the analysis of enzymatic activities in this tissue (Ilic *et al.*, 1999).

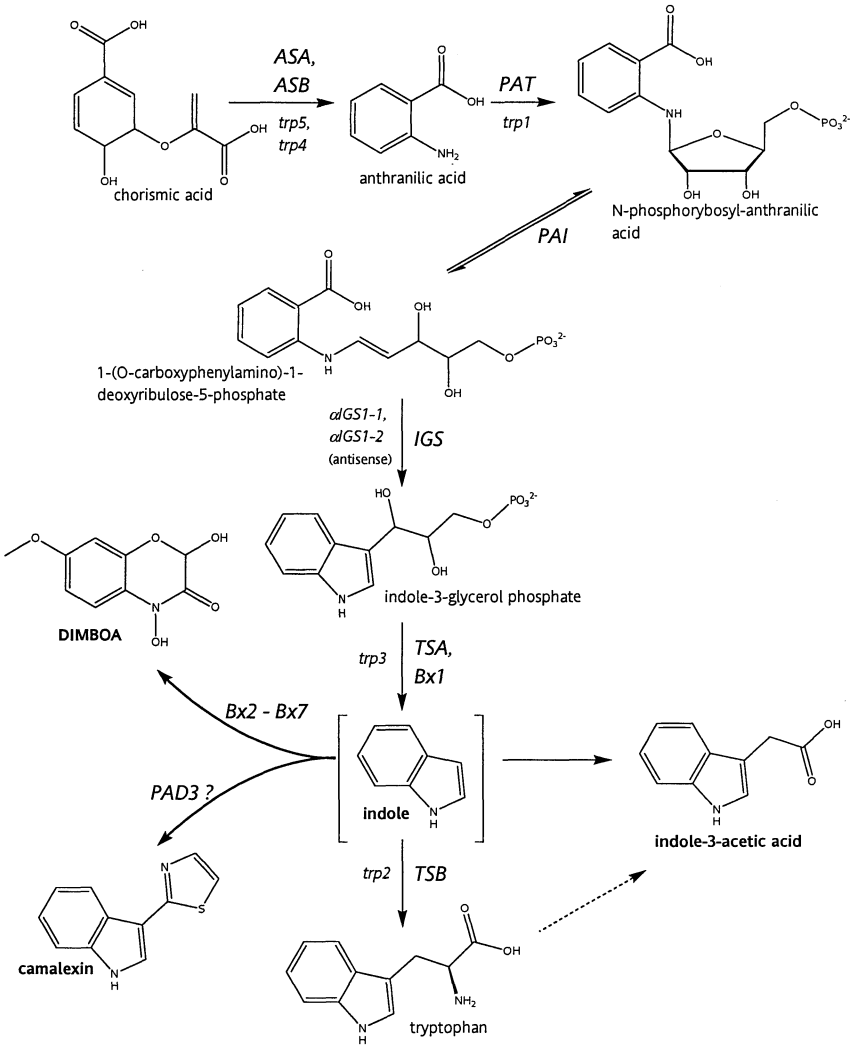

Figure 4: Tryptophan-independent IAA biosynthesis branches from the tryptophan pathway before the TS complex. The proposed indole intermediate is also not likely to be produced by the TSA subunit, but rather by a similar but independent enzyme.

In maize kernel cultures, which are very similar to the developing endosperm, D_5 label from Trp was found to be efficiently incorporated into IAA, and the conversion was not outcompeted by indole. Further labelling experiments with

$^{13}C_6$-glucose and $^{13}C_2$-acetate followed by retrobiosynthetic analysis indicated that the Trp-dependent pathway is a primary source of IAA in this system (Glawischnig *et al.*, 2000). In contrast, enzyme preparations from maize seedlings were shown to synthesize IAA directly from indole, without a Trp intermediate (Östin *et al.*, 1999). The conclusion that can be drawn from these observations is that both IAA biosynthetic pathways can coexist in the same plant and their use is developmentally regulated. Such a concept had been suggested earlier, based on 2,4-D effects on somatic embryogenesis in carrot callus cultures (Michalczuk *et al.*, 1992b). Moreover, it appears that the experimental conditions can greatly affect which of the two pathways is operative (or "detected") in plants. Sztein and co-workers reported recently that in germinating bean axes, removal of cotyledons caused IAA biosynthesis to be switched from the Trp-independent to the Trp-dependent pathway (Sztein *et al.*, 2002). Yet, after a 12-hour recovery period Trp-independent IAA biosynthesis was again restored. Observations that multiple pools of IAA metabolic precursors can exist within the cell, and that exogenous precursors are metabolised preferentially over the endogenous ones (Rapparini *et al.*, 1999), as well as the fact that temperature and presumably other environmental factors can have an effect on which pathway is utilised (Rapparini *et al.*, 2002), certainly do not make solving the IAA biosynthesis puzzle any easier.

Catabolism, conjugation and IAA metabolites

The currently accepted model of IAA catabolism assumes the involvement of processes that modify the chemical structure of the hormone, causing loss of biological activity. Two types of such processes have been proposed in plants: oxidation and conjugation. Of these, oxidation is beyond any doubt an irreversible catabolic process. In analogy to detoxification pathways known from other organisms, IAA conjugation can also be considered a catabolic process. However, it is known that some of the conjugates may be re-hydrolysed in this case, thereby releasing active hormone. Conjugation of IAA involves modification of the side chain of the active hormone. Two classes of conjugates have been described in a variety of plant species:

- ester-types, with the carboxyl group of IAA linked via an oxygen bridge to sugars (for example glucose) or cyclic polyols (like inositol),
- amide-types, with the carboxyl group forming an amide (*e.g.* peptide) bond with amino acids or polypeptides (for reviews see Bandurski *et al.*, 1995; Bartel *et al.*, 2001; Normanly, 1997; Normanly & Bartel, 1999; Reinecke & Bandurski, 1987).

Decarboxylative metabolism of IAA

In decarboxylative catabolism of IAA both the side chain and the indole ring are typically modified. These complex reactions are catalysed by a variety of plant peroxidases, often referred to as "IAA oxidases". The major *in vitro* products are indole-3-methanol (also known as indole-3-carbinol), indole-3-carboxaldehyde (the product of indole-3-methanol reactions with free radicals), 3-methylene-

oxoindole, indole-3-carboxylic acid and 3-methyl-oxoindole (Barcelo *et al.*, 1990; Östin, 1995; Reinecke & Bandurski, 1987). However, these compounds are very infrequently identified as endogenous constituents in plants (Bandurski *et al.*, 1995; Östin, 1995), which may indicate that the pathways synthesizing them are not the major routes of IAA catabolism. Two exceptions are indole-3-methanol, proposed to be a product of the degradation of indole glucosinolates (Chevolleau *et al.*, 1997) and indole-3-carboxaldehyde, which is implicated in the metabolism of phytoalexins (Pedras *et al.*, 2002). However, neither of these pathways seems to be directly involved in IAA metabolism.

In studies with transgenic plants expressing anionic forms of peroxidase in sense and antisense orientations, endogenous levels of IAA were not affected in either case. However, when purified from the plants, the overexpressed protein was able to degrade IAA *in vitro* (Lagrimini, 1991; Lagrimini *et al.*, 1997). Application of exogenous IAA or its analogues caused strong suppression of the expression of anionic peroxidase genes in tobacco (Klotz & Lagrimini, 1996) and also reduced total enzyme activity of the basic peroxidase in protein extracts from *Catharanthus roseus* cell cultures (Limam *et al.*, 1998). Nevertheless, it is interesting that only plant peroxidases are able to decarboxylate IAA using molecular oxygen instead of H_2O_2. Moreover, several amino acid sequence similarities have been found between auxin binding proteins and plant peroxidases (Savitsky *et al.*, 1999). This, however, may simply be due to similarities in the native substrate structure, as mentioned earlier.

Not much is known about decarboxylative IAA metabolism in *Arabidopsis*, although the vast amount of evidence accumulated from other plants suggests it may be similar in most species. Interestingly, activation of anionic peroxidase expression in *Arabidopsis* has been correlated with defects in auxin perception (Mayda *et al.*, 2000), suggesting that this peroxidase isoenzyme (CEVI-1) may be actively involved in auxin homeostasis.

Non-decarboxylative metabolism of IAA

Non-decarboxylative oxidation of the indole ring was found to be a major catabolic pathway in *A. thaliana* (Paper I). Similar pathways have also been described in other plants. However, unlike decarboxylative metabolism, considerable diversity in products is observed between species. In the first committed step of this pathway, either free hormone or its aspartic acid conjugate can be oxidized at the second carbon of the indole ring. The maize oxidation system uses free hormone as a substrate to yield 2-oxoindole-3-acetic acid (OxIAA). This intermediate is subsequently oxidised at the seventh position of the indole part of the molecule, followed by glucosylation of a newly formed hydroxyl group (Nonhebel *et al.*, 1985). While the same pathway also functions in *Pinus silvestris*, other species including *Vicia faba*, tomato (fruit pericarp), carrot and hybrid aspen use IAAsp as the substrate for the first oxidation (Riov & Bangerth, 1992; Sasaki *et al.*, 1994; Tuominen *et al.*, 1994). The second oxidation step in these systems involves formation of the hydroxyl group on the third position of the indole ring, and often glucosylation to produce the 3-O glucoside of OxIAAsp. It has been speculated that, in hybrid aspen, OxIAAsp can be hydrolysed back to

OxIAA (Tuominen *et al.*, 1994), but this has not yet been confirmed. In tomato pericarp tissue, another high molecular weight product has also been described, in which OxIAAsp is linked to the glucose *via* an N-glucoside bond from the indole ring (Östin *et al.*, 1995).

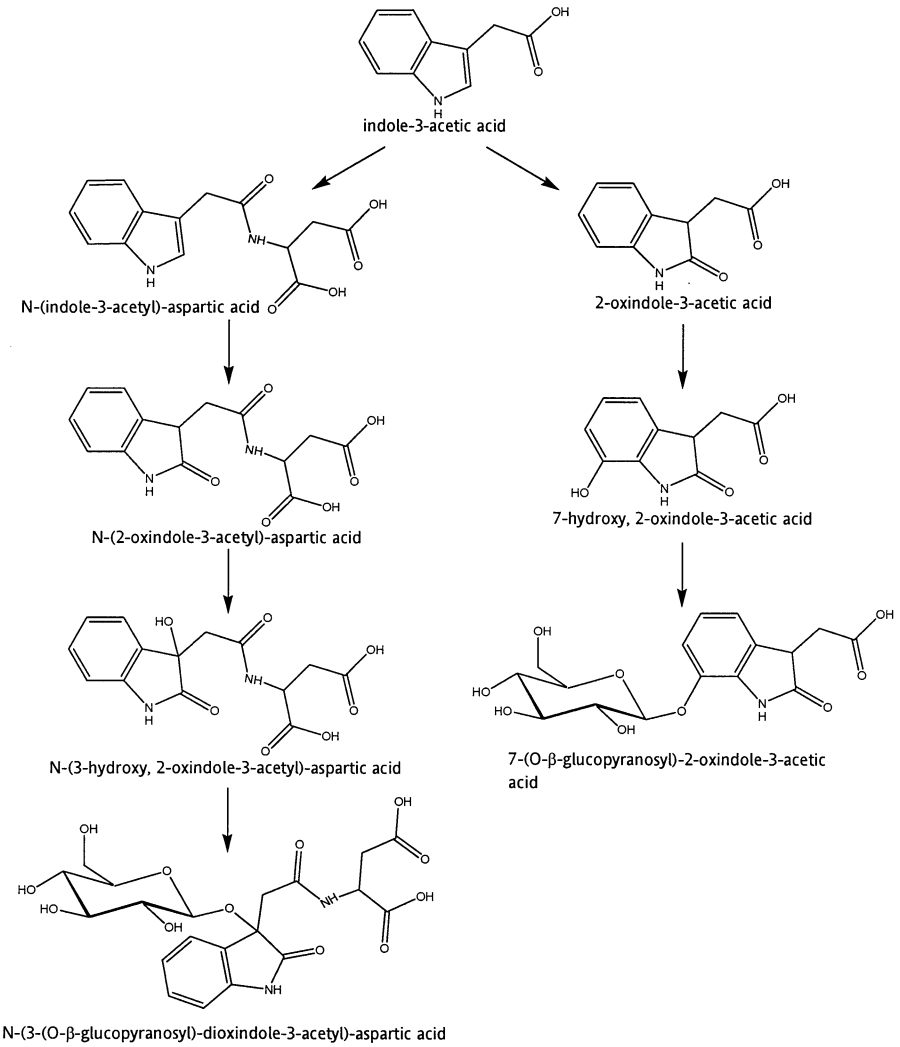

Figure 5: Non-decarboxylative metabolism of IAA in *Vicia faba* (left) and *Zea mays* (right). The hypothesis that IAAsp is the first committed intermediate of the *Vicia faba* pathway is plausible, but has not yet been entirely confirmed.

It is not yet clear whether 2-oxindole-3-acetic acid (OxIAA) or N-(2-oxindole-3-acetyl)-aspartic acid (OxIAAsp) is the initial metabolite for this pathway in *Arabidopsis*. OxIAAsp and N-(3-hydroxy, 2-oxindole-3-acetyl)-aspartic acid (DiOxIAAsp), as well as OxIAA, have been identified in *Arabidopsis* plants incubated with labelled exogenous IAA (see Paper I and the *Current research* section of this manuscript). These findings suggest that the *Arabidopsis* pathway is similar to that described in hybrid aspen, tomato and carrot. However, a major

difference in *Arabidopsis* is the presence of a putative 1-O-(2-oxindole-3-acetyl)-beta-glucose (OxIAGlc), an intermediate to an unidentified product (Paper I). The *Arabidopsis* system seems to be similar to that described in the orange (*Citrus sinensis*) fruit epicarp, where two pathways (from OxIAA leading to DiOxIAGlc and from IAAsp to DiOxIAAsp) seem to operate independently (Chamarro *et al.*, 2001). Irrespective of the specific oxidation products, the results obtained so far in *Arabidopsis* suggest that non-decarboxylative metabolism plays a very important, if not the major, role in maintaining auxin levels (Paper I, Paper VI).

Auxin conjugation

So far, the IAA conjugation pathway has been most intensively studied in maize endosperm by Bandurski and co-workers (review in Reinecke & Bandurski, 1987). In this very specific system, the free IAA is first converted to a "high energy" intermediate, 1-O-(indole-3-acetyl)-beta-D-glucose (1-O-IAGlc). This reversible reaction, catalysed by UDPG-dependent glucosyltransferase (Szerszen *et al.*, 1994, and references therein), favours the formation of 1-O-IAGlc due to the relatively high levels of UDPG present in the tissue and thermodynamically favourable coupling to O-(indole-3-acetyl)-inositol (IAInos) synthesis. The product of the latter reaction is further glycosylated to arabinose (IAInos-Ara) and galactose (IAInos-Gal). A high molecular weight ester conjugate, IAA-glucan, is also formed in this system, presumably from the 1-O-IAGlc.

During seed germination, the level of all IAA ester conjugates decreases, but transport to the shoot has been confirmed only for IAInos and free IAA (Chisnell & Bandurski, 1988). Although the maturing endosperm does not seem to make amide-type conjugates, seedlings are able to synthesize IAAsp from exogenous IAA (Zelena, 2000).

In comparison to the maize endosperm conjugation pathway, little is known about the synthesis of IAA conjugates in other plants. Vegetative tissues of mono- and di- cotyledonous plants are able to form amide conjugates, especially IAAsp and IAGlu, from exogenous IAA. Synthesis of 1-O-IAGlc, IAAla and IAGly has also been reported in such conditions.

From an initial TLC-based screening, it was established that *Arabidopsis* is able to form IAGlc, IAAsp and possibly IAIle/Leu from exogenous IAA (Sztein *et al.*, 1995). Experiments performed using radiolabelled IAA confirmed that it synthesised IAAsp (especially when high amounts of IAA were used for feeding) and IAGlu (Paper I) as well as a previously undescribed amide conjugate, N_{α}-(indole-3-acetyl)-glutamine (IAGln) (Barratt *et al.*, 1999). Initially, neither IAIle/Leu nor IAGlc were found in these experiments, but their presence in *Arabidopsis* was later confirmed (Paper II, Tam *et al.*, 2000). Furthermore, the first *Arabidopsis* gene encoding a protein involved in auxin conjugation (UDPG-dependent IAA glucosyltransferase, a homologue of the maize enzyme) has been identified and cloned (Paper IV). However, the kinetic properties of this enzyme are very different in *Arabidopsis*. The enzyme is much more specific towards IAA, indicating that it may act in an environment with lower IAA levels than in maize endosperm, while its activity is relatively low with other carboxylic acids that are known to be accepted as substrates by the maize enzyme. It is not known which

reaction the synthesis of IAGlc is coupled to in *Arabidopsis*. However, preliminary results of experiments with transgenic plants overexpressing IAGlc synthase indicate that levels of all amide conjugates, and OxIAA in the vegetative tissues, decrease in these plants, (R. Jackson *et al.*, in press, see also the *Current research* section of this manuscript).

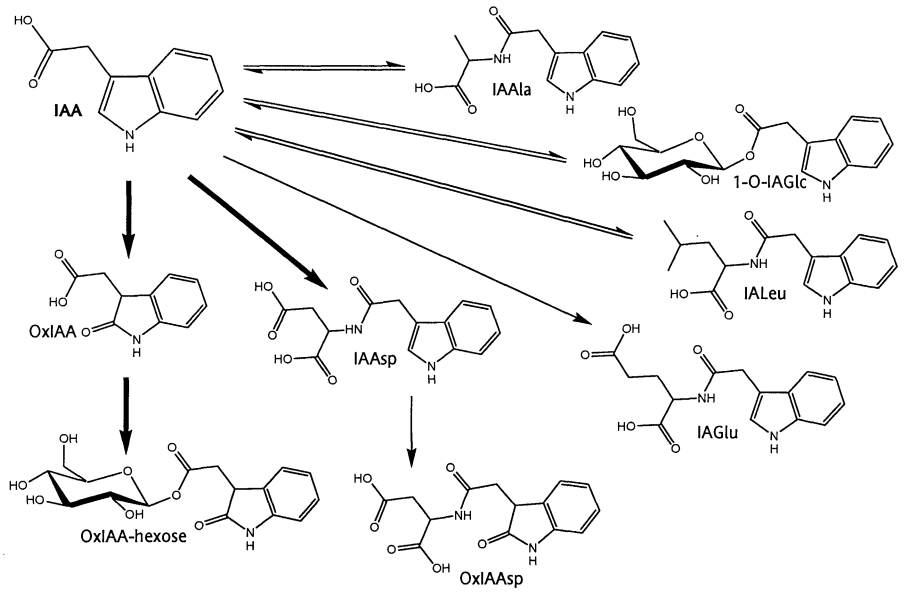

Figure 6: Major pathways of IAA catabolism and conjugation in *Arabidopsis thaliana*. The glutamine conjugate is not shown, but is most probably synthesized either directly from IAA or from IAGlu. In seeds, high molecular weight peptide conjugates, also not shown in the figure, constitute the majority of amide-linked IAA.

The first quantitative measurements of the concentration of two amide conjugates (IAAsp and IAGlu) and IAGlc in *Arabidopsis* indicated that they occur at very low levels (Tam *et al.*, 2000). While this is not entirely surprising, especially since both IAAsp and IAGlc can be considered as intermediates rather than products, the levels of conjugates measured by the "total IAA" method are usually very high. A GC-MS screening revealed the presence of an additional conjugate, IAAla and confirmed that IALeu is an endogenous constituent in *Arabidopsis*. However, both of these newly discovered conjugates occur at concentrations even lower than those of IAAsp and IAGlu (Paper VI). This opens the possibility that high molecular weight IAA conjugates (either peptide or oligosaccharide), which are difficult to analyse by TLC or GC/MS methods, are present in *Arabidopsis* in large quantities. The occurrence of peptide/protein types of IAA conjugates in plants was first shown in *Phaseolus vulgaris* (Bialek & Cohen, 1986) and their role in homeostatic hormone control was subsequently established (Bialek & Cohen, 1992; 1989a). Antibodies generated against a 3.6 kDa IAA-containing polypeptide from *Phaseolus* were used to screen for similar proteins in *Arabidopsis*. This experiment resulted in the identification of the IAP1 protein, and subsequent cloning of its gene (Walz *et al.*, 2002). IAP1 and related

polypeptides are proposed to constitute the major fraction of amide-type IAA conjugates in *Arabidopsis*.

The conjugate hydrolysis process is believed to be one of the most important processes controlling auxin homeostasis, and a large amount of evidence has been obtained suggesting that both amide and ester type conjugates can be hydrolysed back to a free hormone. Amide conjugates can serve as slow-release auxin sources in plant tissue cultures (Magnus *et al.*, 1992) and in high concentrations some of them can have the same phytotoxic effect as high concentrations of IAA (Slovin, 1997). A screening for plants insensitive to IAA amide conjugates led to the discovery of the first *Arabidopsis* conjugate hydrolase, encoded by the *ILR1* gene (Bartel & Fink, 1995). The enzyme's amino acid sequence was found to be similar to procaryotic aminoacylases and hippuricases. ILR1 has a strong substrate preference for IALeu and IAPhe. Using a similar approach another hydrolytic enzyme, encoded by the *IAR3* gene, has been identified (Davies *et al.*, 1999). Activity of the IAR3 protein is highest with IAAla, a conjugate well known for its auxin-like activity. Overall, six different amide conjugate hydrolases are thought to exist in *Arabidopsis* (Davies *et al.*, 1999).

Other indole-containing compounds in plants

Tryptophan-derived glucosinolates

Glucosinolates are a class of thioglucosides found exclusively in members of the order *Capparales*, consisting of fifteen plant families. Species containing glucosinolates include *A. thaliana* as well as several agriculturally important plants, like rapeseed, cauliflower, cabbage and broccoli. A number of excellent reviews on different features of glucosinolate physiology, genetics and biochemistry have been published recently (Chen & Andreasson, 2001; Mithen, 2001; Wittstock & Halkier, 2002), and early reactions of their biosynthesis have been discussed in this chapter in the framework of auxin biosynthesis. Nevertheless, some additional aspects of their structure, metabolism and examples of indole-derived glucosinolates will be briefly discussed here.

To date, over 100 different glucosinolates have been characterized in plants, 24 of which have been found in *A. thaliana* ecotype Columbia. All glucosinolates share the basic thioglucoside structure, but have very diverse aglycones. The aglycone moiety can originate from any one of seven amino acids: alanine, leucine, isoleucine, valine, phenylalanine, tyrosine or tryptophan, as well as from chain-elongated amino acids derived from methionine and phenylalanine. Based on this property, glucosinolates can be classified as aliphatic, aromatic or indole-derived. The side-chain structure can be additionally altered by a variety of secondary modifications, giving rise to the observed diversity of these compounds in plants. However, some types of glucosinolates can only be found in specific families or even species (see Fahey *et al.*, 2001, for an excellent review).

Biosynthesis of glucosinolates (Figure 7A) starts with oxidative decarboxylation of the parental amino acid to form an aldoxime, in a reaction typically catalysed by a highly specific cytochrome P450. The aldoxime is then converted by another high affinity P450 system to an *aci*-nitro compound, serving as an acceptor for the nucleophilic thiol-group donor. The resulting conjugate is cleaved by C-S lyase to

produce a thiohydroxyimic acid derivative, which is subsequently S-glucosylated by the action of UDPG-dependent glucosyltransferase. The product of the latter reaction is sulfonated by sulfotransferase to form a glucosinolate, which can be subjected to further side-chain modifications. The enzymes catalysing reactions downstream of the *aci*-nitro intermediate are proposed to have rather broad substrate specificity, and the final steps of glucosinolate biosynthesis presumably use the same enzymatic apparatus, regardless of the amino acid from which the substrate originated.

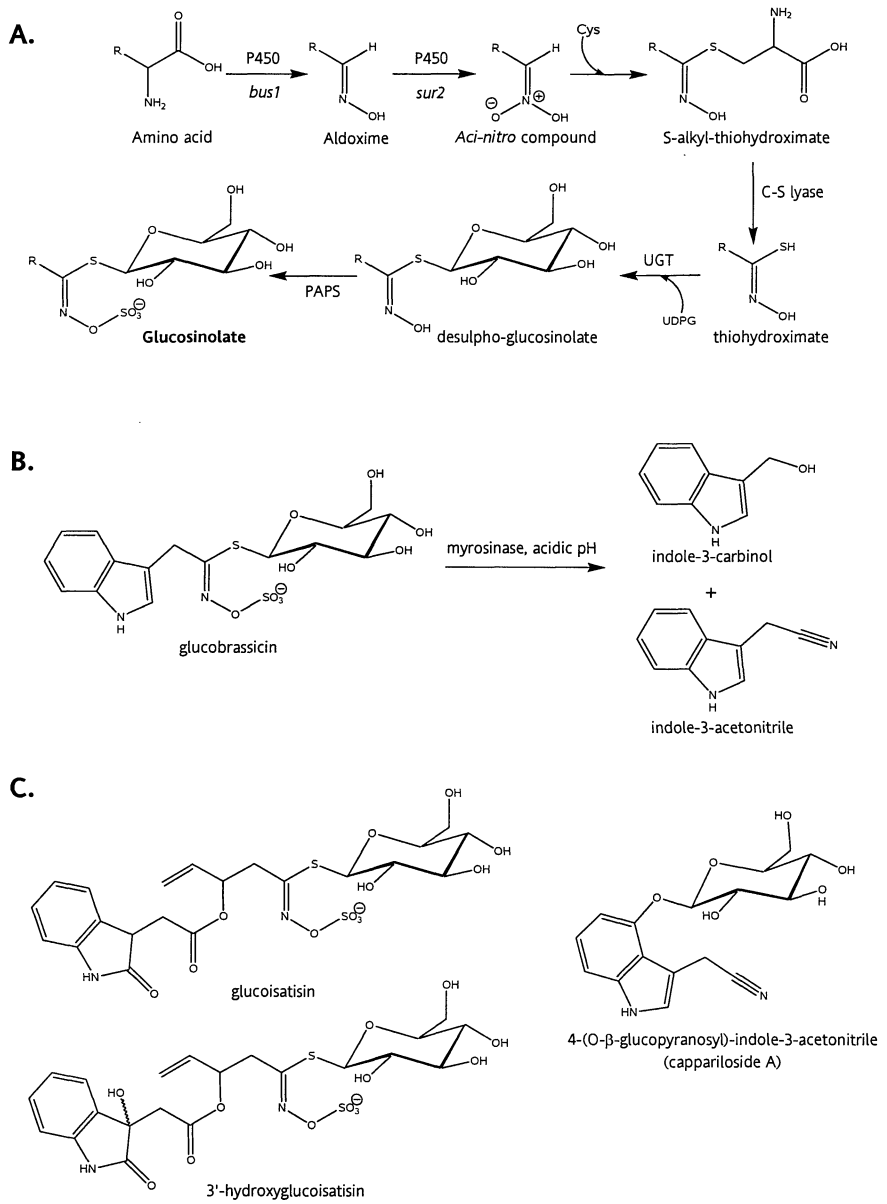

Figure 7: Indole glucosinolates and derived compounds: biosynthesis and degradation (see descriptions in text).

Tryptophan-derived glucosinolates are certainly of interest for auxin biochemistry. The initial steps of the indole glucosinolate biosynthesis and putative Trp-dependent auxin biosynthesis pathways are shared (Paper II, Bak et al., 2001; Hull et al., 2000). Although these two pathways branch at the level of indole-3-acetaldoxime (IAOx), disruption of metabolite flow into the glucosinolate pathway can result in increased auxin production (Paper II). Interactions between glucosinolates and auxins are further complicated by the fact that one of the proposed IAA precursors, indole-3-acetonitrile (IAN) may potentially originate from both dehydration of IAOx and the enzymatic breakdown of primary indole glucosinolate (glucobrassicin, indole-3-ylmethyl glucosinolate or GBS, the structure and probable degradation pathway of which is shown in Figure 7B) (Chevolleau et al., 1997). High levels of glucobrassicin were recently reported in Arabidopsis siliques and mature seeds (Petersen et al., 2002), correlating with high levels of IAA in the same tissues (Kowalczyk & Sandberg, unpublished – see *Current research* section). Even though the link between IAA and GBS levels in seeds may be purely coincidental, the occurrence of the "parental group" of both indole and aliphatic glucosinolates in seeds and siliques indicates that active biosynthesis is taking place in these organs, and GBS synthesis may also provide intermediates for IAA production.

Although glucobrassicin and its 4-methoxy-, 1-methoxy-, and 4-hydroxy-derivatives are the major indole glucosinolates, at least two examples of glucosinolates that are not derived directly from Trp and yet contain an indole moiety have been identified (Frechard et al., 2001). Both of these compounds were isolated from woad (*Isatis tinctoria*), a relatively common Mediterranean plant known to contain large amounts of indole glucosinolates. Its aglycones consist of 2-hydroxybuten-3-yl side-chains, identical to those of progoitrin, a methionine-derived hydroxyalkyl glucosinolate also found in *Arabidopsis* (Reichelt et al., 2002). However, in *Isatis* the hydroxyl group of the side-chain is esterified with OxIAA, yielding glucoisatisin, or with DiOxIAA in 3'-hydroxyglucoisatisin (chemical structures are shown on the left in Figure 7C). Nothing is known about the origin of the oxoindole part of these molecules, however it is likely to be derived from IAA.

Also interesting, but most probably related to glucosinolate breakdown products rather than to glucosinolates *per se*, are IAN glycosides isolated from fruits of *Capparis spinosa* (Calis *et al.*, 1999). The aglycone component in these substances is indole-3-acetonitrile, connected to the fourth carbon of the indole ring with glucose *via* an O-glycoside bond (details of the chemical structure are presented on the right in Figure 7C). Again, the synthesis routes and origins of these compounds are unknown.

Phytoalexins and other plant defence compounds

Phytoalexins are small organic molecules with antimicrobial properties that are synthesized by plants in response to pathogen attacks. This very diverse group covers compounds derived from flavonoids, terpenoids and indole. Interestingly, all 25 phytoalexins isolated so far from plants belonging to the *Cruciferae* family

contain an indole ring in their chemical structure (Pedras *et al.*, 2000). To date however, only one phytoalexin, camalexin (3-thiazol-2'-yl-indole, CMX), has been identified in *Arabidopsis* (Tsuji *et al.*, 1992).

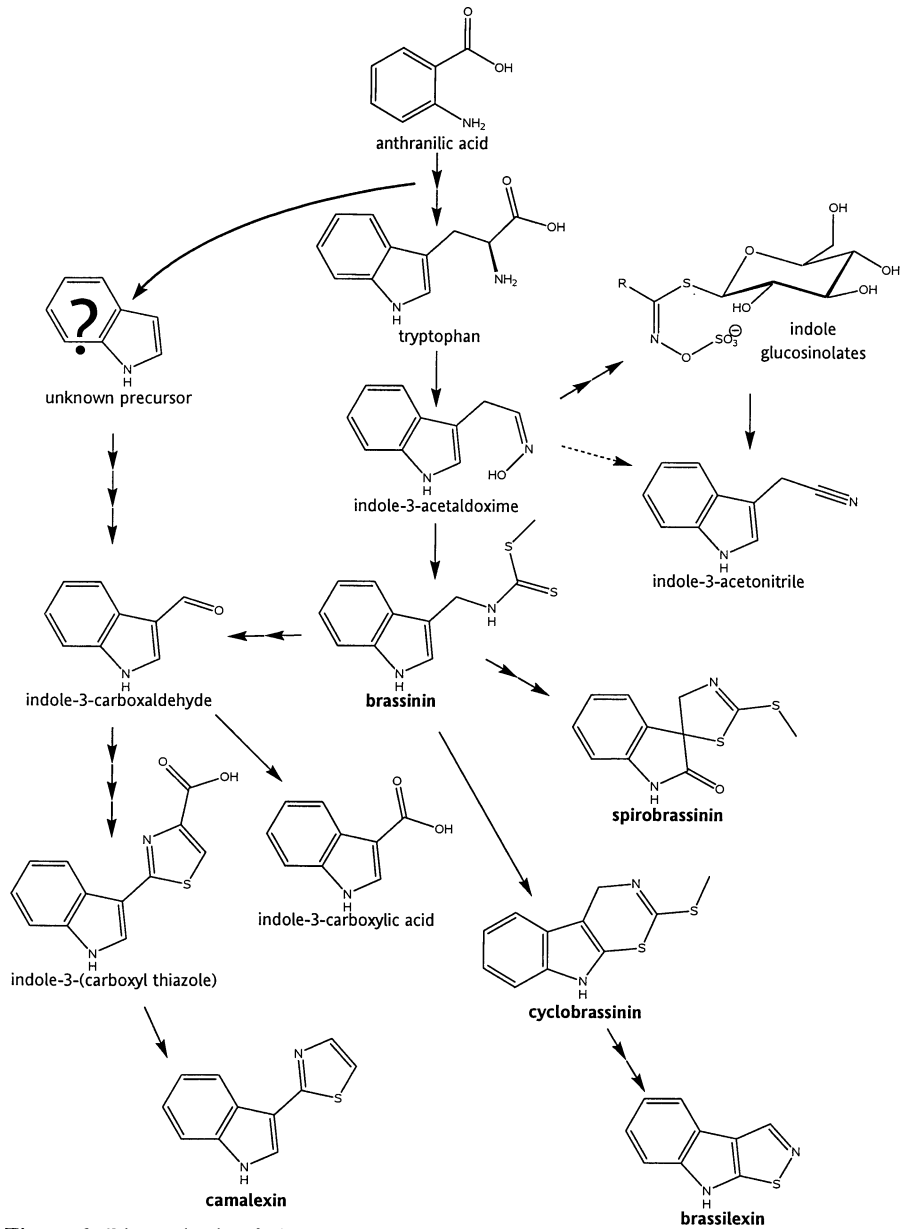

Figure 8: Biosynthesis of phytoalexins in *Arabidopsis thaliana* and related species (details in text).

It has been proposed that camalexin biosynthesis occurs through the condensation of indole-3-carboxaldehyde with cysteine, formation of a thiazole ring via cyclization and subsequent decarboxylation (Browne *et al.*, 1991; Zook &

Hammerschmidt, 1997). Although indole-3-carboxaldehyde (IAld) can be produced from IAA in reactions catalysed by "IAA oxidase", a P450 cytochrome protein that presumably forms IAld from an unknown intermediate, was recently cloned (Zhou *et al.*, 1999). Camalexin is even more intriguing in the context of its potential metabolic precursors. *In vivo* labelling studies have shown that instead of tryptophan, anthranilate (Tsuji *et al.*, 1993) and indole (Zook, 1998) are used for CMX biosynthesis. These remarkable findings prove that the origin of the indole ring in natural compounds does not necessarily have to involve Trp as an intermediate.

Furthermore, Trp-independent biosynthesis is also well established for a *Gramineae*-specific group of plant defence compounds - benzoxazinoids. The initial step of their biosynthesis involves the production of indole from indole-3-glycerol phosphate, catalysed by the Bx1 enzyme, which is similar to the alpha subunit of Trp-synthase, but acts independently of the Trp-synthase complex. Indole is then used for the synthesis of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its 7-methoxy- derivative (DIMBOA) in a pathway involving four oxidations and a ring expansion. All reactions downstream of the indole formation are catalysed by P450 cytochromes from the CYP71C family (Frey *et al.*, 1997; Glawischnig *et al.*, 1999). In maize, genes (*Bx1* to *Bx5*) encoding enzymes from the DIBOA pathway form a cluster on chromosome IV and their expression is most likely synchronized. Since benzoxazinoids occur in vacuoles as glucosides, the *Bx8* gene encoding glucosystransferase is also present in this cluster (von Rad *et al.*, 2001).

Other members of the *Cruciferae* family seem to contain much larger assortments of phytoalexins than *Arabidopsis*. One of the major phytoalexins from *Brassica* species is brassinin, an indole derivative structurally related to the aglycone of glucobrassicin, a glucosinolate mentioned in the previous section. Both compounds naturally occur within the same plant species (with the notable exception of *Arabidopsis*, which apparently lacks brassinin), and their levels have been reported to increase simultaneously in response to stress. Furthermore, the proposed precursor of both of these compounds is tryptophan. Labelling experiments using D_4-IAOx have indicated that, at least in roots of *Brassica rapa*, IAOx serves as a precursor for indole glucosinolates as well as brassinin and the related spirobrassinin (Pedras *et al.*, 2002). Further experiments in the same system demonstrated that the brassinin biosynthesis pathway operates independently of glucobrassicin, thus showing that IAOx is a branching point not only for glucosinolates and presumably auxins, but also for phytoalexins. It was also shown that in infected stems of *Brassica junicea*, brassinin was actively metabolised, yielding indole-3-carboxaldehyde and its oxidised derivative, indole-3-carboxylic acid.

Alkaloids

The family of monoterpenoid indole alkaloids consists of around 1800 very diverse compounds found in plants and animals. A model plant for indole alkaloid biosynthesis studies is *Catharanthus roseus,* which produces numerous pharmacologically important compounds, including vindoline and ajmaline as well

as the bisindole alkaloid vinblastine. Indole alkaloids occurring in plants are derived from two precursors: the indole ring comes from tryptamine, while the terpenoid part originates from secologanin, an iridoid glucoside synthesized from geraniol. Tryptamine is produced from tryptophan in a reaction catalysed by a pyridoxal-quinoprotein, tryptophan decarboxylase (TDC). This activity has been isolated from various plants and is known to have high specificity towards tryptophan and its analogues (for a review see Facchini *et al.*, 2000). The first reaction in the biosynthesis pathway of indole alkaloids is strictosidine synthase (STR)-catalysed condensation of tryptamine with secologanin to produce strictosidine. Strictosidine is a central biosynthetic intermediate, thought to be a precursor for all other major indole alkaloids found in *C. roseus*: tabersonine, ajmaline, vindoline and catharanthine. Although little is known about the early biosynthetic reactions leading to catharanthine and tabersonine, synthesis of vindoline has been relatively well investigated. Conversion of tabersonine to vindoline starts with hydroxylation at C-16 catalysed by a P450-dependent tabersonine-16-hydroxylase (T16H). The newly-formed hydroxyl group is subsequently methylated by a SAM-dependent O-methyltransferase (OMT), followed by hydration of the 2,3-double bond and N-methylation of the indole ring by N-methyltransferase (NMT). The final steps of biosynthesis include hydroxylation at C-4 by an oxoglutarate-dependent dioxygenase (D4H) and acetylation of that hydroxyl group by acetyltransferase (DAT). The bisindole alkaloid vinblastine is then synthesized by condensation of vindoline and catharantine in a reaction catalysed by a non-specific peroxidase (for more details see Chou & Kutchan, 1998; Facchini, 2001).

A handful of genes encoding enzymes from this pathway have been cloned and *in situ* RNA hybridisation has been used to analyse their expression. In young leaves, transcripts of the genes encoding enzymes of early biosynthetic steps, *i.e.* TDC and STR, were found to be restricted to the upper and lower epidermis. Conversely, mRNAs for D4H and DAT, the late biosynthesis enzymes, were found only in the specialised cells (so-called idioblasts) of the palisade and spongy mesophyll, and in cells cross-connecting the two types of mesophyll (so-called lactifers) (St-Pierre *et al.*, 1999). The same pattern was established in the old leaves, although the levels of transcripts were much lower in these organs. Based on the hybridization data, it was also shown that meristems of various actively growing organs are able to express *TDC* and *STR* genes, although again exclusively in the epidermis layer. These findings are very interesting in the context of the potential tryptamine involvement in IAA biosynthesis. The indole alkaloids are intensively produced in young leaves and meristems, the locations where IAA synthesis allegedly takes place. However, since in *C. roseus* only one gene encoding TDC has been found, IAA biosynthesis *via* the tryptamine intermediate would have to occur only in the epidermis. Alternatively, tryptamine or its metabolites would have to be transported to other cells that can produce IAA, as in synthesis of indole alkaloids.

Objectives and current research

Objectives

The major objective of this work was to obtain biochemical insights into the metabolism of indole-3-acetic acid in the model plant, *Arabidopsis thaliana*. To accomplish this, the following goals were set:

- to investigate the metabolism of exogenous IAA in *Arabidopsis*, in order to establish the major pathways of catabolism and conjugation (Paper I)
- to develop methods for quantitative analysis of IAA catabolites and precursors (Paper VI)
- to investigate the effect of various mutations on IAA metabolism (Papers II, III and IV)
- to develop tools to investigate proteins involved in IAA metabolism (Paper V)

Current research

This chapter of the introduction describes results from experiments that must be labelled as "work in progress". Nevertheless, from the author's perspective it is worth presenting them, because they extend the scope of previously published materials included in this thesis.

Experimental

Plant material and growth conditions

Wild-type *A. thaliana* plants used in this study were of ecotypes Columbia or Wassilewskija. Seeds were sterilised with 90% ethanol for 2 min and dried on sterile filter paper. Before germination, they were cold treated for 48 hrs and then grown on either agar-solidified Murashage-Skoog medium (M-S) or in M-S liquid cultures for 12 or 14 days, respectively, with long day light conditions and growth temperatures in the range of 22-24°C. IAA biosynthesis intermediates were analysed in *sur1* (Boerjan *et al.*, 1995) and *sur2* (Delarue *et al.*, 1998) mutants using wild-type Wassilewskija as a control.

Analysis of IAA metabolism was performed on mutants of the *axr* series (*axr1-1*, *axr2*, *axr3-1* and axr4: Hobbie & Estelle, 1995a; Leyser *et al.*, 1996; Lincoln *et al.*, 1990; Wilson *et al.*, 1990), two ethylene mutants (*eto2*, *ctr1* (Kieber *et al.*, 1993; Vogel *et al.*, 1998)) and two mutants with perturbed IAA-conjugate hydrolysis (*ilr*, *ilr* x *iar* (Bartel & Fink, 1995; Davies *et al.*, 1999)). Wild type *Arabidopsis* of the Columbia ecotype was used as a control in these analyses, as well as in IAA UGT overexpression experiments.

Measurement methodologies

Quantification of endogenous IAA metabolites and precursors was performed using internal standard isotope dilution techniques. In this approach, heavy labelled standards of the analytes are added to the samples at an early stage of

preparation, prior to extraction. Currently only a few labelled auxin-related standards are available commercially: [indole-$^{13}C_6$]- and [indole-D_5]-IAA, [indole-D_5]-Trp, [benzyl-$^{13}C_6$]-anthranilic acid and [2,2'-side-chain-D_2]-tryptamine (all from Cambridge Isotope Laboratories). The other internal standards were synthesized from labelled IAA. Synthesis of labelled amide conjugates was performed using the method of Ilic and co-workers, except that [indole-$^{13}C_6$]-IAA was used as a starting material and the purification scheme was simplified (Paper VI; Ilic *et al.*, 1997). Oxidized IAA was made using the method of van de Weert *et al.* (1998), with modifications (Paper VI).

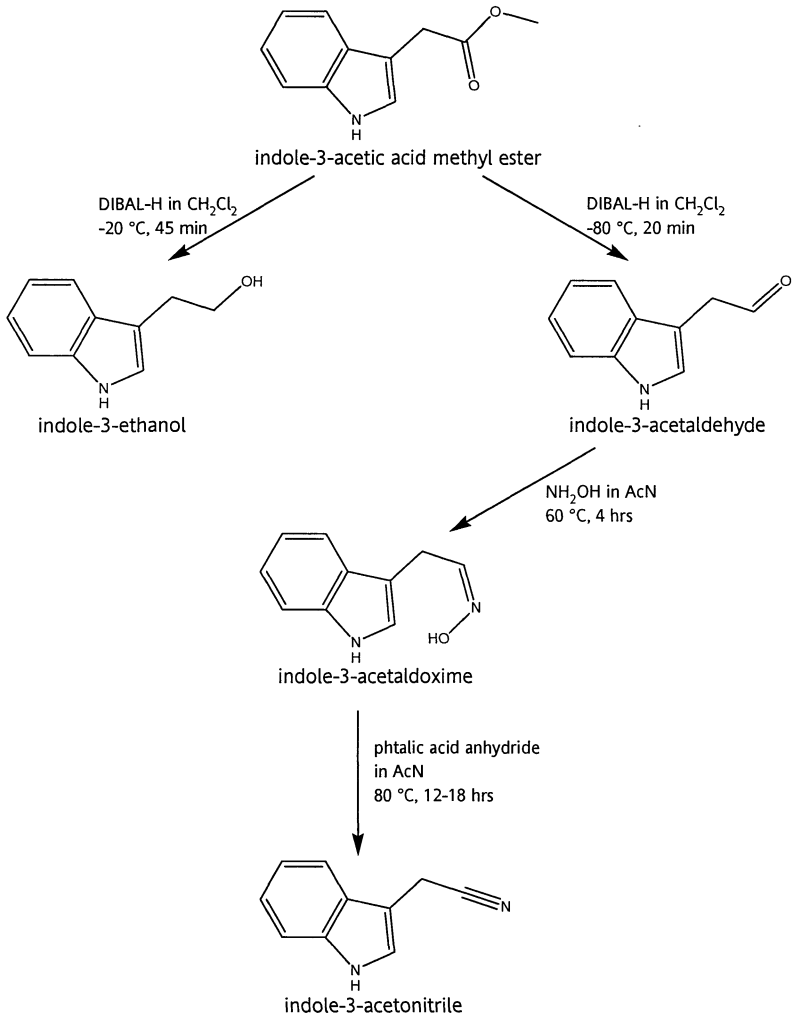

Figure 9: Chemical synthesis scheme of IAA metabolic precursors. Both [indole-D_5]- and [indole-$^{13}C_6$]-labelled IAA methyl esters were used for the synthesis

For synthesis of the IAA precursors a new method was developed, starting from the methyl ester of labelled IAA and resulting in labelled IAEt, IAAld, IAOx and IAN (Figure 9). Due to the relatively easy non-enzymatic decomposition of IAOx into IAAld, two different labels (D_5 for IAOx and $^{13}C_6$ for IAAld) had to be used

for these compounds, to allow compensation to be made for this conversion if it occurred during sample preparation. Of the proposed IAA biosynthetic precursors for Trp-dependent pathways (Figure 3), only IPyA and tryptamine N-hydroxide internal standards were not synthesized and, consequently, these compounds were not measured. Although D_5-IPyA synthesis was attempted using the method of Tam and co-workers (Tam & Normanly, 1998), we did not produce a reasonable yield of this compound.

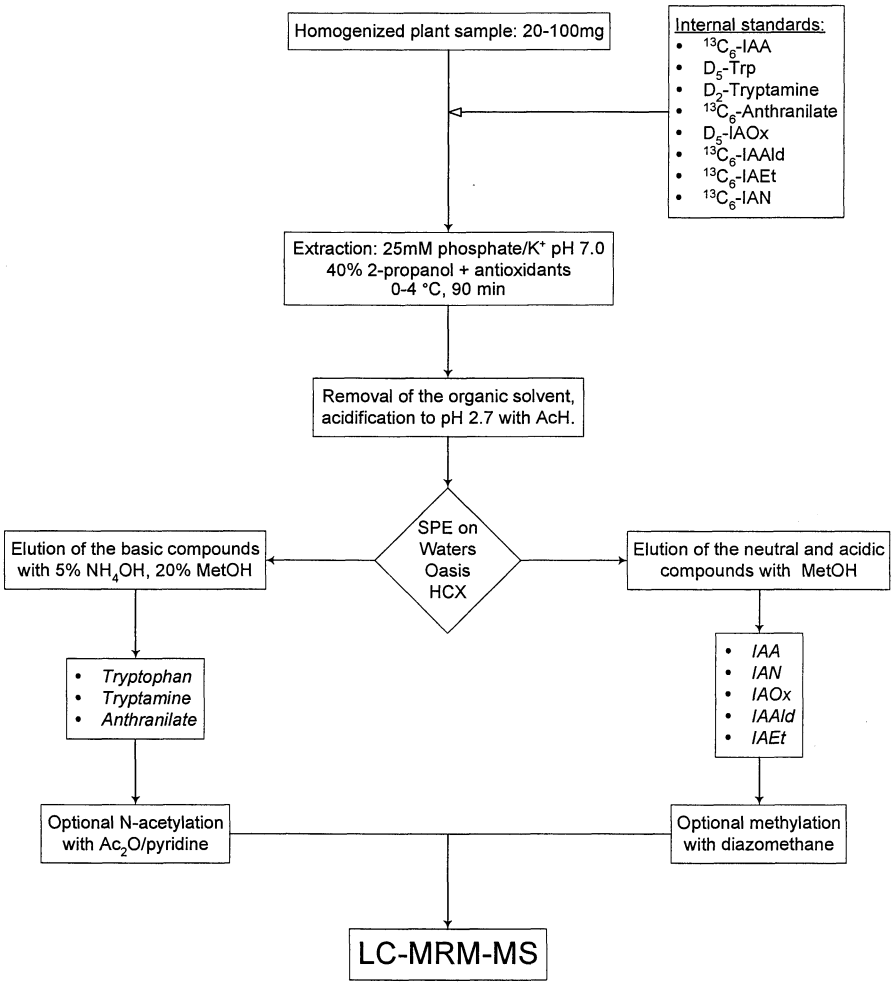

Figure 10: Sample preparation for IAA precursor analysis. Neutral/acidic and basic fractions from solid phase extraction (SPE) can either be combined and analysed together or analysed separately. All derivatization steps are optional, and the method was shown to work satisfactorily without them.

The sample preparation techniques preceding the measurements were designed for simplicity and to ensure good recoveries of the measured compounds rather than for maximal purity. In addition, the cost of the entire purification procedure was seriously considered. Extraction was performed at pH 7.0 using buffered 2-propanol and optimised for recoveries. This required extended extraction times, so

care had to be taken to avoid enzymatic degradation or oxidation of the analytes. The final step of purification for the IAA metabolites and precursors involved solid phase extraction on hydrophobic IST Isolute Env+ or mixed-mode Waters Oasis HCX cartridges, respectively. After that, compounds could be derivatized, if required, and were ready for analysis (Figure 10, Paper VI).

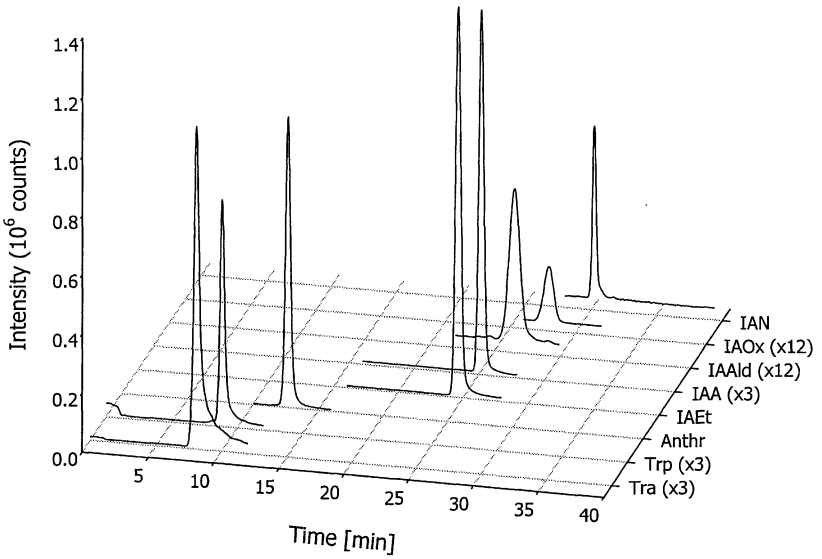

Figure 11: Example of the measurement of IAA precursors and related metabolites by LC-MRM-MS. Compounds (500 pg each) were separated by liquid chromatography and the reaction from the [MH^+] to the most prominent daughter ion was monitored in MRM mode. Measurement groups used were based on retention time (see description in Paper VI).

All the measurements were performed using liquid chromatography-mass spectrometry (LC-MS) with positive mode electrospray ionization (ESI+). This method offers several advantages over the more commonly used gas chromatography-mass spectrometry (GC-MS). First, compared to GC-MS, less derivatization is required, because analytes do not have to be volatile. If a derivatization procedure is applied, the aim is rather to improve chromatographic resolution of the analytes and increase sensitivity towards compounds that are not easily ionisable in ESI. For quantification of IAA metabolites (Paper VI), methylation was used to increase sensitivity, but the IAA precursors and IAGlc were analysed without derivatization. Second, ESI is a soft ionisation technique that does not cause excessive fragmentation during the ionisation of analytes, and gives quite intense parent ion signals. This is important for MS/MS quantification methods, such as multiple reaction monitoring (MRM), because decreased fragmentation of the parent ion can improve yields of daughter ions and, thus, increase sensitivity. Finally, compared to GC-MS, more material per injection can be analysed in LC-MS. A typical injection volume in the classical capillary GC-MS analysis is 1µl out of a 5-10µl sample. Of course, this is quite reasonable as long as such a volume contains more than the quantification limit of the target analyte. However, for many compounds of low abundance, achieving this

concentration may require the preparation of samples from large amounts of material and therefore the use of complex, "scaled-up" and time-consuming purification procedures (see Tam *et al.*, 2000, for an example). Such procedures are unfortunately required sometimes, because capillary GC columns are easy to overload with contaminating substances, which in turn affects the chromatography as well as detection of the analyte. In contrast, the injection volume in LC-MS is limited in practice only by the volume of the autosampler loop and large volumes do not necessarily result in poor chromatographic resolution if, as in this study, trace enrichment is used.

The method for quantifying IAA metabolites (Paper VI) was fully validated and its precision was regularly tested with each experiment performed. The method for IAA quantifying precursors has not yet been validated. Nevertheless, it provides good separation of analytes (Figure 11), and relatively good detection for most of them, with quantification limits in the low-to-medium pg range.

Results and Discussion

General considerations

Auxin research has reached the stage where molecular genetics and physiology data greatly outweigh biochemical information in both quantity and quality. This is particularly true for studies on IAA metabolism. Despite recent progress in identifying the genes involved, such as *ilr* (Bartel & Fink, 1995), *iar* (Davies *et al.*, 1999), *yucca* (Zhao *et al.*, 2001), and *nit1-nit4* (Bartel & Fink, 1994; Bartling *et al.*, 1992), the biochemical details of IAA catabolism and biosynthesis are still unclear. Lack of high quality analytical methods can be partly blamed for this state of affairs. The majority of studies mentioned above in the review section of this introduction relied on measurements of either a combination of free, ester and "total" IAA contents or just one of these variables. For reasons explained in Paper VI, the "total IAA" method has relatively limited scope, and although it is convenient to use, it may not always produce reliable results. Moreover, even if free, "ester" and "total" IAA are measured (Baldi *et al.*, 1989; Bialek & Cohen, 1989b), the entire branch of non-decarboxylative IAA metabolism remains unanalysed and unknown. The situation is even more unsatisfactory for IAA biosynthesis intermediates, where only tryptophan, anthranilate and IAN are measured on a more regular basis (Ilic *et al.*, 1996; Michalczuk *et al.*, 1992a; Rapparini *et al.*, 1999). Regardless of the fact that quantification methods for some other IAA precursors have been published (Cooney & Nonhebel, 1991; Prinsen *et al.*, 1997; Tam & Normanly, 1998), they are very rarely used.

The approach presented here - measurement of a number of metabolites in the same sample, is derived from the concept of "metabolomics" (see Fiehn, 2002; Fiehn *et al.*, 2000, for more details). However, the aim of this research is targeted analysis of a small number of IAA-related pathways rather than non-biased study of the entire metabolism. Thus, some important differences between the two methods have to be emphasized. The major objectives of the analytical methods described here were to obtain good precision and sensitivity of measurements. The former was achieved by using separate internal standard and separate calibration for each compound measured, while generic metabolomic analysis uses one

internal standard for a number of compounds within the retention time frame. Thus, in non-biased metabolomics, calibration curves can be trusted only in the case of fully identified analytes, for which standards are available. Applying such calibration to the unknown compounds may cause quantification errors that are difficult to correct and poor precision. To improve sensitivity, MRM measurements were used instead of scanning throughout an arbitrary range of *m/z* values. An attempt to increase the selectivity of the measurements by grouping metabolites (see Paper VI for details) resulted in an additional improvement in sensitivity. The concentrations at which IAA metabolites are found in *Arabidopsis* make them extremely difficult to detect in small amounts of plant material if full scan MS analysis is used. The GC-MS full scan screen we performed to identify potential IAA metabolites required as much as 80 g of plant material to obtain good quality mass spectra (Paper VI), and the GC-MS SIM (single ion monitoring) quantification method proposed for IAA conjugates needed around 10 g (Tam *et al.*, 2000). In contrast, the LC-MRM-MS methods presented here and in Paper VI required 20-100 mg of plant tissue. Further development of these techniques should allow us to decrease the amounts required to just 2-10 mg.

It must be stressed that the majority of results presented here reflect static, pseudo-steady state levels of IAA metabolites in the entire *Arabidopsis* plants. Even though the concept of steady state over the entire multicellular organism is at best dubious, we assume that cells and perhaps specific tissues can and do maintain such metabolic states. However, instruments to measure them at the tissue level are neither readily available nor sensitive enough for detailed analysis as yet. Investigation of the pathway dynamics can evidently be done using the whole or part of organisms, but the biochemical significance of such analysis should again be carefully considered. Analysis of pathway dynamics usually involves a range of *in vivo* labelling studies, carried out in a targeted or non-biased manner. Targeted analysis is typically done with labelled precursors of the metabolite of interest, as in the experiments with ^{15}N-anthranilate, D_5-Trp and D_4-IAOx mentioned in the review part of this introduction. Non-biased studies use precursors that can incorporate label into a wide array of metabolites, such as D_2O and $^{13}C_2$-acetate, and have been successfully employed in plant hormone research (see, for example, Cooney & Nonhebel, 1991, Sitbon *et al.*, 2000, and Glawischnig *et al.*, 2000). Both approaches should lead to a calculation of biosynthetic rates that portray the dynamic state of the analysed pathway. Application of methods described here for the dynamic analysis of IAA-related pathways in *Arabidopsis* is currently in progress. However, regardless of the obvious shortcomings of analysis based solely on steady-state metabolite levels, it can provide useful insights into the biochemical phenotype of various mutants. Four examples of such experiments will be presented here.

Analysis of IAA metabolism in Arabidopsis mutants

Shortly after we identified the major IAA metabolites in *Arabidopsis* (Paper I), we decided to use the exogenous [1'-^{14}C]-IAA labelling technique to investigate IAA metabolism in auxin-insensitive mutants. For a number of reasons this effort was not very successful, and did not produce any useful quantitative data. Although exogenous IAA metabolism studies are certainly very helpful for metabolite

identification, they provide little understanding of how auxin metabolism functions at physiological concentrations of the hormone. The focus of the research was then shifted to measurements of the endogenous metabolites, which were presented in Paper VI. Nevertheless, questions remained about whether defects of auxin perception and/or signalling affect IAA metabolism. Therefore, we attempted to address these issues once again, this time using a newly developed quantification method. For this, samples were prepared from agar-grown seedlings, 12 days old, and five metabolites (OxIAA, IAAsp, IAGlu, IAAla and IALeu) in addition to free IAA were analysed, as described in Paper VI. The results of these measurements are presented in Table I.

Although differences in metabolite levels between the mutants are clearly visible, general trends are more difficult to discern. To solve this problem, principal component analysis (PCA) was applied.

Table I: Levels of IAA metabolites in various *Arabidopsis thaliana* mutants. Values are given in pg per mg fresh weight, and presented as means ± SD from three independent samples.

Name:	IAA	OxIAA	IAAsp	IAGlu	IAAla	IALeu
WT	17.96±0.8	31.73±1.6	6.8±0.1	0.85±0.1	0.20±0.01	0.16±0.01
axr1-3	21.33±2.0	46.19±2.2	6.2±0.1	1.00±0.1	0.13±0.01	0.09±0.01
axr2	21.96±4.6	58.78±1.9	14.2±0.1	1.48±0.2	0.13±0.01	0.10±0.02
axr3-1	27.60±2.5	69.85±3.6	9.0±0.1	1.16±0.1	0.12±0.02	0.07±0.01
axr4-1	10.98±0.8	43.21±3.6	3.6±0.1	0.41±0.1	0.19±0.02	0.16±0.02
ilr	11.73±3.2	5.64±1.0	3.2±0.1	0.35±0.1	0.12±0.01	0.08±0.01
ilr x iar	12.40±3.2	5.94±0.2	4.2±0.1	0.59±0.1	0.15±0.03	0.09±0.03
eto2	48.93±12	48.38±2.6	4.0±0.1	0.64±0.2	0.13±0.01	0.08±0.01
ctr1	14.57±1.4	49.75±3.6	4.8±0.1	0.47±0.1	0.13±0.02	0.10±0.02

PCA is a bi-linear modelling technique that is frequently used to reduce the number of data if correlations are present between the variables (see Miller & Miller, 2000, for a general introduction). It is used to reveal systematic, dominant types of variation between the samples by means of a special class of latent variables, the so-called principal components (PCs). The PCs reflect the largest eigenvalues of the co-variances in the data, so that the first few PCs provide adequate description of the variation in the whole dataset, and PC1 accounts for more of the variation than any of the other PCs. It should be emphasised that in most cases individual PCs do not correspond directly to individual physico-chemical variables. Instead, two or more PCs together describe how the physico-chemical parameters vary in the samples.

PCA applications in plant biochemistry and physiology are rare and have so far been limited mostly to the analysis of metabolomics datasets that contain much larger numbers of variables than the study presented here. In this experiment, we could expect at least some correlation between variables, because they reflected concentrations of metabolites derived from the same compound, which was also measured. The PCA on this particular dataset was performed using the levels of IAA metabolites found in discrete samples rather than their mean value in a given mutant, to test how well similar samples could be classified. Results of the analysis (see score plot of PC1 vs. PC2 in Figure 12) indicate that auxin metabolism varies in different mutants sufficiently to allow them to be classified

according to this property. The internal structure of the dataset was further tested by partial least squares projection on the latent structures discriminant analysis (PLS-DA), whereby one sample from each putative class was removed and subsequently fitted with every class model using PLS-DA. In all cases, the best fit was for the class from which the sample was taken (data not shown).

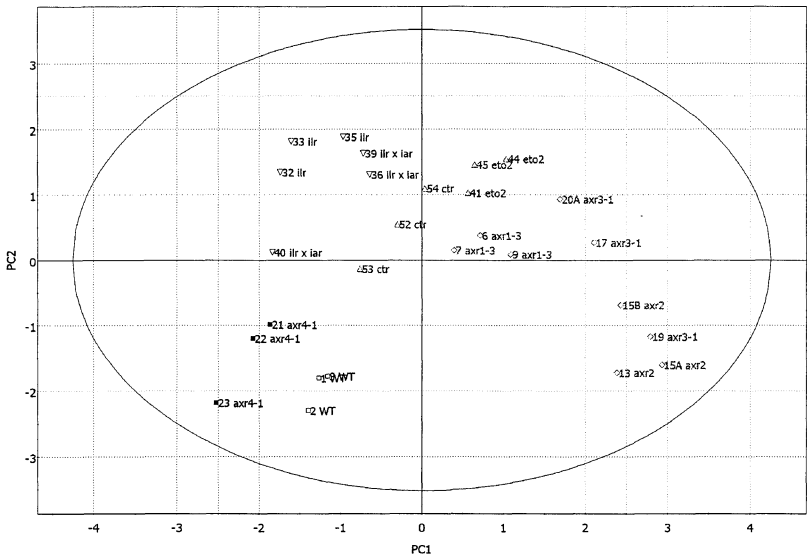

Figure 12: Score plot of PC1 vs. PC2 for the PCA analysis of IAA metabolism in *Arabidopsis thaliana* mutants.

Both PC1 vs. PC2 (Figure 12) and PC1 vs. PC3 (data not shown) score plots indicate that the group containing *axr4* is more similar to the wild type than to the other *axr* mutants. This is presumably because the levels of IAA and its amide conjugates in *axr4* are in roughly the same range as in the wild-type, while *axr1 – axr3* have higher levels of OxIAA, IAAsp, IAGlu and IAA as well as slightly decreased levels of IALeu and IAAla. In contrast, the class containing the *ilr* mutant can be characterised by a large decrease in all the metabolites, together with a slight decease in the free hormone level in comparison to the wild-type. The ethylene mutants form a separate group, with *eto2* containing much more IAA and OxIAA, but significantly decreased levels of IAAla and IALeu. The *ctr1* mutant shares a similar metabolic phenotype, but has free IAA levels closer to those of the wild-type.

Linking these observations with the physiology of analysed mutants is nevertheless rather difficult. In the case of the *ilr* class, the reduction in free IAA could be explained by the lack of hydrolytic enzymes with specific activities towards IALeu and IAAla. Surprisingly, however, accumulation of these two metabolites was not observed. On the other hand, based on double mutant analysis, *axr4* is known to differ from *axr1* (Hobbie & Estelle, 1995b). Gravitropic growth of the root could be restored in *axr4* by the application of

NAA (Yamamoto & Yamamoto, 1999), which suggests that the axr4 protein may be involved in auxin transport rather than auxin signalling. However, this requires further investigation, because NAA is also able to restore apical dominance in *axr3* (Cline *et al.*, 2001), which encodes a known member of the Aux/IAA gene family (see the Auxin signal transduction section of this introduction). Although differences at the protein level between *axr1* and *axr2* have been reported (Leymarie *et al.*, 1996), in the context of IAA metabolism these two mutants appear to differ only in the magnitude of their response, with *axr2* showing more severe phenotypic divergence from wild-type. Nevertheless, the finding that defects in auxin signalling resulting in auxin resistance also induce changes in IAA metabolism is very interesting. Except for *axr4*, the mutants have higher levels of free hormone and consequently higher levels of its major metabolites. The latter finding suggests that the catabolism of IAA may not be under the control of the mutated signalling pathway, while the former suggests that decreased sensitivity to a hormone may perhaps be partially counteracted by an increase in its internal concentration.

Both ethylene mutants show similar patterns of IAA metabolism, which is generally consistent with the biochemical and physiological functions of the mutated genes. The ethylene overproduction in *eto2* leads to a range of responses that are constitutive in *ctr1*. Quite a lot is known about the way auxins interact with ethylene in the regulation of developmental processes (Collett *et al.*, 2000; Pitts *et al.*, 1998; Swarup *et al.*, 2002) and at the gene expression level (Woeste *et al.*, 1999). In particular, genes encoding the key enzyme in ethylene biosynthesis: ACC synthase, are induced by auxin and some auxin responses are thought to be mediated by ethylene (Abel *et al.*, 1995a; Peck & Kende, 1995; Yi *et al.*, 1999). Moreover, some of the auxin resistant mutants are also resistant to ethylene, for example *axr2* (Wilson *et al.*, 1990), and presumably *aux1* (Pickett *et al.*, 1990). However, the effects of ethylene on auxin metabolism are largely unknown. Based on the evidence presented here, it seems that non-decarboxylative catabolism of IAA may be important for auxin/ethylene interactions, since in both mutants OxIAA levels are increased, while the levels of IAA-conjugates are decreased in comparison to the wild-type. Yet, to fully understand the changes of IAA metabolism in these mutants, biosynthetic rates for the free hormone and its metabolites will also have to be compared.

IAA metabolites during seed germination

The hypothesis that hydrolysis of auxin conjugates is a primary source of the free hormone during seed germination is largely accepted and has considerable experimental support. As mentioned earlier, in *Phaseolus vulgaris*, *A. thaliana* and presumably other dicotyledonous plants, high molecular weight amide conjugates have been proposed to be the major seed storage forms of IAA (Bialek & Cohen, 1986; Walz *et al.*, 2002). In maize, ester type conjugates: IAInos, its glycosides and probably IAA-containing β-1,4-glucan, have been shown to serve this function (Chisnell & Bandurski, 1988). In this context, we wanted to investigate IAA metabolism during seed germination. In particular, we wanted to examine if low molecular weight amide conjugates are also hydrolysed during the germination of seeds in *Arabidopsis*, and to test the effect of light on the

conjugates' hydrolysis. For these purposes, sterile seeds were cold-treated for 24 hrs, and then incubated at room temperature for an additional 24 or 48 hrs imbibition period. After that, they were germinated on sterile filter paper and grown in either light or dark conditions for 2-4 days. Collected samples were analysed as described in Paper VI, and a representative set of results from this analysis is shown in Figure 13.

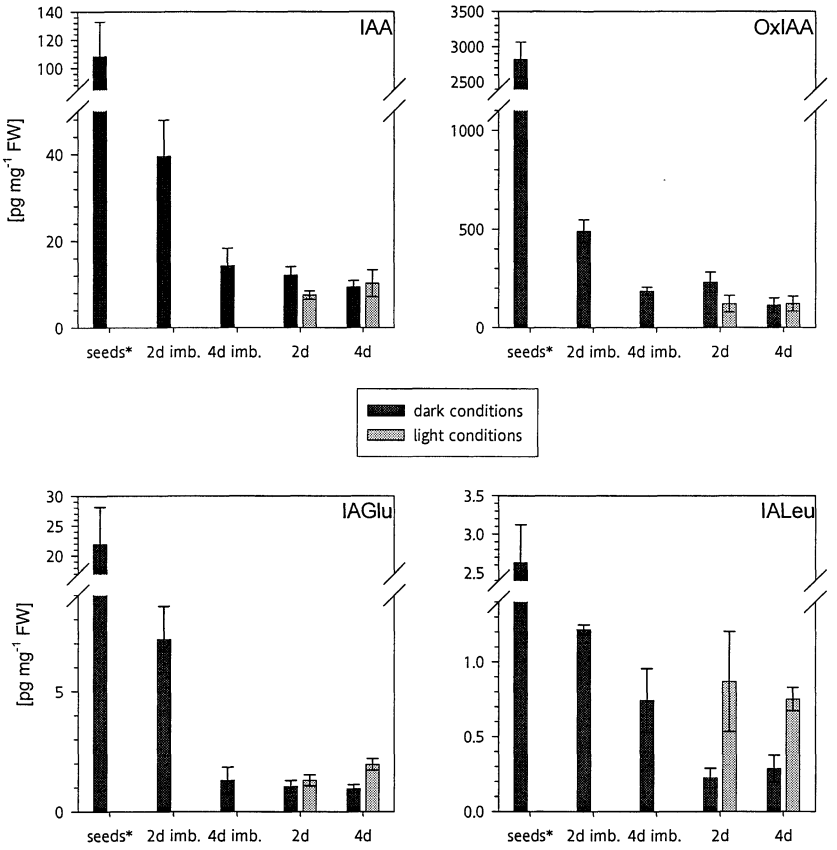

Figure 13: IAA and representative metabolites during the germination of seeds in light and dark conditions. Bars represent means from five independent samples, error bars represent standard deviations.

Again, it has to be stressed that these measurements reflect concentrations in the entire seedling, and the patterns in Figure 13 may not necessarily be true for specific organs or parts of the plant. Steep reductions in levels of all the metabolites are clearly visible, but this is most likely at least partially due to the dilution of the seeds' metabolite pools during imbibition. Even though hydrolysis of amide conjugates (IAGlu and IALeu) cannot easily be observed, it cannot be categorically excluded, as the decrease in levels does not follow very precisely the increase in volume/biomass. The concentration of free hormone in the seeds is also rather intriguing, because assuming a 4-5 fold increase in volume/biomass occurs during the imbibition period, this amount of IAA would probably be

sufficient for their developmental requirements, without any need for supplemental hydrolysis of IAA conjugates. For IAA, IAGlu, IALeu (Figure 13), and IAAsp (data not shown) slight accumulations can be observed after four days of germination in the light, while levels of these metabolites steadily decreased in the dark. This indicates that *de novo* synthesis of IAA and its metabolism may start to take occur at this point, although this finding will require further confirmation from labelling studies. Light is known to affect exogenous IAA metabolism in maize seedlings (Zelena, 2000). In this system, IAAsp synthesis was strongly promoted by light in the mesocotyl, which was linked with inhibition of growth due to the depletion of free IAA.

Overexpression of IAGlc synthase in Arabidopsis

1-O-IAGlc was first isolated and characterised by M.H. Zenk from leaves of the monocotyledonous plant *Colchicium neaoplitanum* (Zenk, 1961). Its role in IAA metabolism in maize endosperm is very well recognized (see references in the review section of this *Introduction*) and synthesis of this compound from endogenous and exogenous hormone has been reported from other plants (Sitbon *et al.*, 1993; Sztein *et al.*, 1995; Tam *et al.*, 2000). Genes encoding UDPG-dependent glucosyltransferase, which catalyses the synthesis of IAGlc, have been cloned from maize (Szerszen *et al.*, 1994) and *A. thaliana* (Paper IV). Although the compound itself is typically considered an IAA ester conjugate, it really represents a special type of alkyl-acetal high-energy intermediate used for synthesis of other metabolites. Similar intermediates have been observed in other biosynthetic pathways, for example in the synthesis of sinapoylmalate and sinapoylcholine (Lim *et al.*, 2001; Lorenzen *et al.*, 1996). So far, only one reaction directly involving IAGlc is known – the synthesis of IAInos in maize (Kesy & Bandurski, 1990). Nevertheless, it has been speculated that IAGlc could also be a precursor of amide-type conjugates. This hypothesis was initially tested by Oetiker and Aeschbacher using an exogenous IAA labelling technique in a temperature-sensitive mutant of henbane (*Hyoscyamos muticus*), but the results suggested that IAGlc is not a precursor to IAAsp in this system (Oetiker & Aeschbacher, 1997)

To examine potential functions of IAGlc in *Arabidopsis*, concentrations of IAA metabolites were analysed in plants with ectopic expression of a native IAGlc synthase gene from the CaMV 35S promoter (R. Jackson *et al*, in press). Three lines of transgenic plants with different levels of IAGlc synthase gene expression were investigated according to the procedure described in Paper VI. As seen in Figure 14, levels of all measured metabolites were significantly decreased, and in most cases, this correlates well with the level of IAGlc synthase expression. Furthermore, endogenous concentrations of 1-O-IAGlc show a pattern opposite to those of amide conjugates and OxIAA – *i.e.* they increase proportionally with increases in transcript levels. Surprisingly, however, IAA levels show a similar correlation (Figure 15A).

At this point, it should be noted that measuring 1-O-IAGlc in plant samples is rather challenging. The compound is very unstable, and during sample preparation it can undergo a variety of modifications, including spontaneous and β-glucosidase catalysed hydrolysis, or pH and ionic strength dependent isomerisation to 4-O-

and 6-O-IAGlc. In addition, the presence of NH_4^+ ions can result in aminolysis of IAGlc to form indole-3-acetamide, while in weak alkaline or acidic solutions containing alcohols trans-esterification may occur. IAGlc-mediated esterification of glycerol has been observed before (Kowalczyk & Bandurski, 1990), and in the case of methanol, this process can lead to the formation of the methyl ester of IAA. Furthermore, commonly used esterification procedures, such as methods involving BSTFA/pyridine or acetic anhydride/pyridine, can also cause serious breakdown of IAGlc to yield IAA.

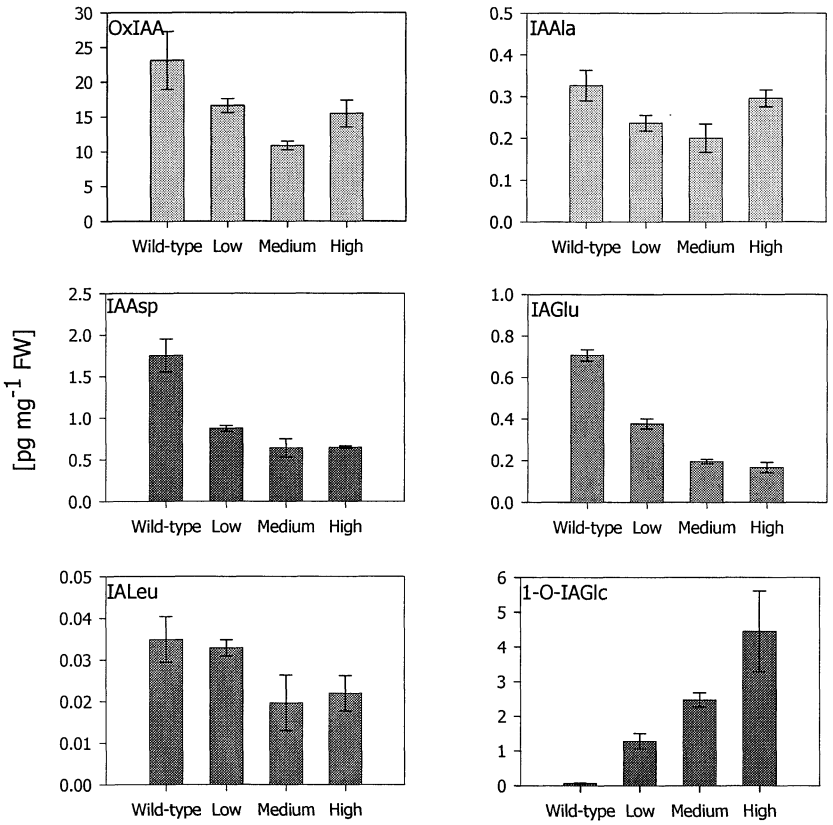

Figure 14: Levels of IAA metabolites in the plants overproducing 1-O-IAGlc. Bars represent means from three independent samples, error bars are SDs. Labels on the X axis indicate the level of IAGlc synthase gene expression.

Given the analytical problems associated with 1-O-IAGlc measurements, the observed increase in IAA levels in the lines transformed with the IAGlc synthase gene could be due to improper sample preparation procedures, resulting in the degradation of IAGlc. To test this hypothesis, we used two different internal standards: [indole-D_4]-IAA and 1-O-[indole-$^{13}C_6$]IAGlc, allowing us to adjust IAA measurements to compensate for putative IAGlc breakdown. The IAGlc to IAA conversion during the samples' purification was found to contribute only around 5-8% of the free hormone concentration detected, indicating that some

other phenomena must be responsible for the increased IAA levels in IAGlc overproducers.

Since synthesis of IAGlc is an easily reversible, near-equilibrium reaction, its direction should depend on the concentrations of both substrates and products. Theoretically, it is possible that if cells are significantly depleted of free IAA and/or UDPG, the reaction will occur in the reverse direction, as long as enough IAGlc had been accumulated. High levels of UDPG and the coupling reaction of IAInos synthesis prevent this in maize endosperm. However, in *Arabidopsis* conditions favouring the reversed reaction may occur in cells that either do not produce sufficient IAA or use large amounts of UDPG (for example in sucrose synthesis).

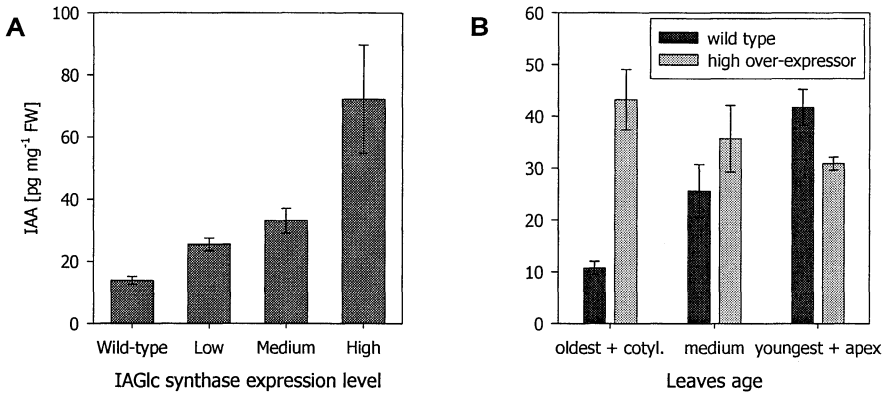

Figure 15: Levels of IAA in the entire seedlings (A) and in the leaves of different ages (B) of wild type and IAGlc overproducing plants. Bars represent means of three independent samples and are corrected for IAGlc hydrolysis during sample preparation. Error bars represent SD.

This possibility was tested by measuring IAA levels in leaves of different ages (Figure 15B). In plants overexpressing IAGlc synthase at the highest level, the apical part containing the youngest leaves have reduced concentrations of the free hormone, which progressively increase as the leaves age, and finally peak in cotyledons. An exactly opposite pattern was seen in the wild-type plants. Thus, the auxin-deficient phenotype observed in plants overproducing IAGlc may be explained by decreased IAA concentrations in the apex, while at the entire seedling scale the level of the hormone is actually higher than in wild-type. Moreover, increased synthesis of IAGlc apparently results in severe deregulation of the usual catabolic pathways, based on the reductions in the levels of OxIAA, IAAsp and IAGlu, which are typically major IAA metabolites in the wild-type. It could be argued that amide conjugates do not accumulate in IAGlc over-producers, so 1-O-IAGlc cannot be their direct metabolic precursor. However, it is possible that IAGlc conversion to amide conjugates requires an "activation step", catalysed by an enzyme that is not present at that particular stage of *Arabidopsis* seedlings' development.

Mutants overproducing IAA

While the major objective of this work was to elucidate key aspects of IAA catabolism and conjugation in *Arabidopsis*, an auxiliary method for the analysis of tryptophan-derived biosynthetic intermediates of IAA is also currently being developed. This technique was used to characterise the biochemical phenotype of two mutants known to overproduce auxin: *sur1* and *sur2*. Some aspects of auxin metabolism in the latter mutant were published in Paper II. However, the analysis was done using a different methodology, and at that time the aim was rather to investigate alleged defects in IAA conjugation (Delarue *et al.*, 1998).

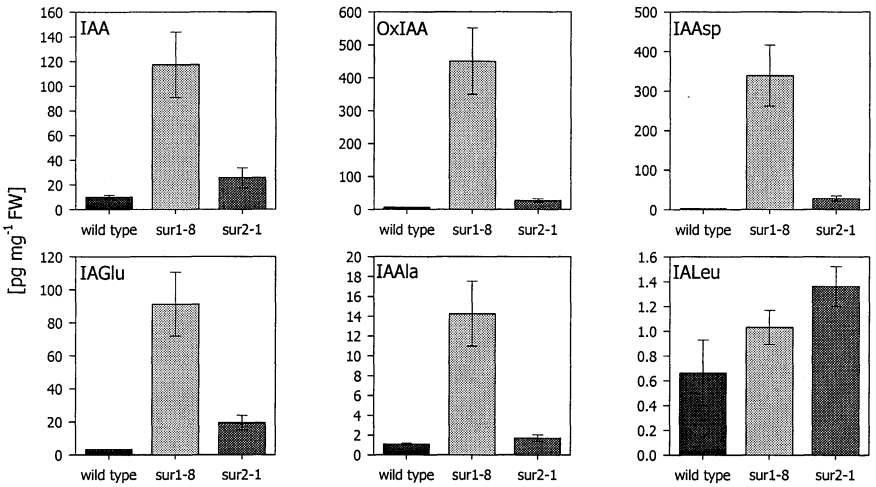

Figure 16: Endogenous concentrations of IAA and its metabolites in the wild-type and mutants overproducing IAA. Bars represent means from five independent samples, error bars show standard deviations.

In this experiment, auxin catabolites were first re-analysed using the method described in Paper VI. As expected, both mutations resulted in increased levels of IAA conjugates and its major catabolite (Figure 16). This effect is more prominent in *sur1*, and correlates with the fact that this mutant contains significantly more free IAA than *sur2*. Even though the levels of IALeu in *sur2* were previously reported to be lower than in the wild-type (Paper II), reduced levels were not observed in this case, presumably because of differences in plant material and growth conditions. In addition, the previous IALeu analysis involved quantities close to the detection limit for the GC-MS-based measurement method, which could cause quantification errors. Nevertheless, in both mutants the effect of auxin overproduction on its metabolism was quite clear. The same plant material was subsequently used for the measurement of IAA precursors according to the procedure shown in Figure 10. The levels of anthranilate, tryptophan, IAOx, IAEt and IAN detected are presented in Figure 17. Four additional compounds were analysed (IAA, tryptamine, IAAld and IBA), but for reasons described below, results of these determinations are not shown in this figure. Free IAA measurements were used to check the quality of the analysis, and the concentrations were almost identical to those obtained previously (Figure 16).

Not even trace amounts of tryptamine were detected in *Arabidopsis* (Figure 18), although the calculated detection limit was around 1 pg, thus placing reliable quantification at the 3-4 pg level. Conversely, the detection limit for IAAld was too high (around 1 ng) to allow reliable quantification. Nevertheless, IAAld was observed in *sur1* and *sur2* samples, and work is in progress to improve the method's sensitivity for this compound. Finally, although at present we do not have an internal standard for IBA, measurement of the compound was attempted using $^{13}C_6$-IAA methyl ester as an internal standard, which gives a response of similar range and has a retention time very close to that of IBA. Unfortunately, the precision of these measurements was very poor. Combined with relatively low levels of IBA in the analysed samples, this resulted in very inaccurate quantification. However, no significant changes in endogenous IBA concentrations were observed in the analysed mutants (data not shown).

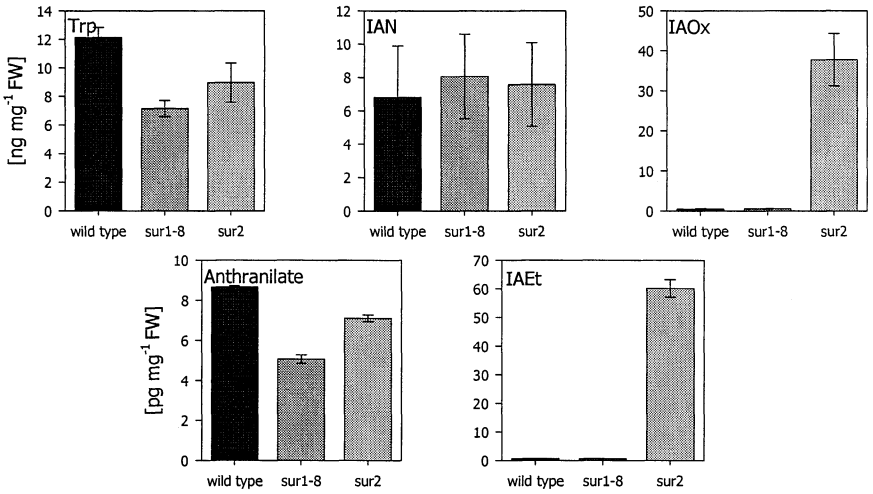

Figure 17: Analysis of IAA metabolic precursors in wild-type and mutants overproducing IAA. Bars represent means from three independent samples, error bars show standard deviations.

The analysis of *sur2* largely confirmed all the findings previously published (Paper II, Bak *et al.*, 2001). The mutant accumulates IAOx, as well as its putative metabolism product, IAEt (Pedras *et al.*, 2002). Both of these compounds were also found at much lower concentrations in the wild-type and *sur1*. This indicates that in *sur1* the IAOx pathway is most likely not involved in the IAA overproduction and is probably mainly involved in glucosinolate biosynthesis, as it is in the wild-type. Both *sur1* and *sur2* have slightly increased levels of IAN, although the statistical significance of these results is very questionable in this case. In *sur1*, the change in IAN level does not correspond to that of IAA, again suggesting that this compound may not be directly involved in auxin overproduction. Since tryptamine was not detected, two biosynthetic routes could potentially be responsible for IAA overproduction in *sur1*: the Trp-independent or the IPyA-mediated pathway. Evidence in favour of the latter has been presented (Gopalraj *et al.*, 1996), but not confirmed. It should be noted that in *sur1* and *sur2*,

soluble tryptophan and anthranilate are decreased in similar proportions, roughly correlating with the increase in IAA level. Although this might be a coincidence, it may also indirectly indicate that the Trp-dependent pathway contributes to auxin overproduction in both mutants, as undeniably proven for *sur2*.

The observation that tryptamine was not present in the developmental stage at which samples were collected has rather interesting implications for a recently proposed IAA biosynthesis pathway involving the YUCCA protein (Zhao *et al.*, 2001). The stage of development may be of particular importance here, because auxin measurements and other experiments on YUCCA were originally carried out on seedlings that were six days old, while the measurements in this study were on seedlings that were 12-14 days old. However, the hypothesis that YUCCA takes part in auxin synthesis in *Arabidopsis* is based on three lines of evidence. Probably the most important is the observation that *YUCCA* overexpressing plants display a variety of auxin overproduction-related phenotypes, which correlates with the increased endogenous IAA concentration in these plants. The second relevant finding is that *YUCCA* overexpressors are resistant to a toxic analogue of tryptophan, 5-methyl-tryptophan, which allegedly indicates that YUCCA protein participates in a Trp-dependent pathway. Finally, YUCCA has been shown to catalyse N-hydroxylation of tryptamine *in vitro* (Zhao *et al.*, 2001).

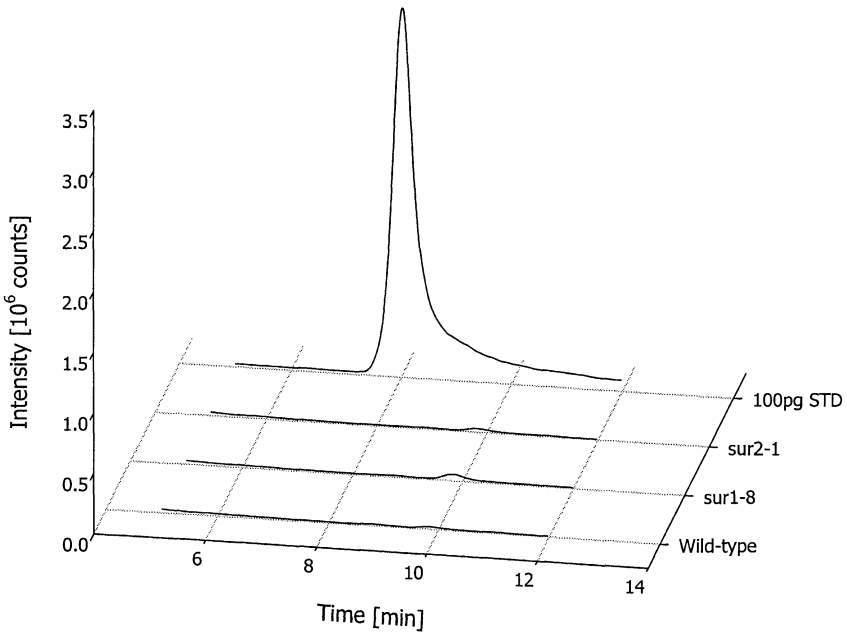

Figure 18: Tryptamine analysis in the wild-type and IAA overproducing mutants. The first three chromatograms represent transition from the $[MH^+]$ ion at *m/z* 161 into the major daughter ion at *m/z* 144 (loss of ammonia, typical for primary amines in electrospray ionisation). The last chromatogram presents such transition for internal standard trace (*m/z* 163 into 146), and was taken from the wild-type sample (first chromatogram).

Although the hypothesis that YUCCA is involved in auxin synthesis received rather enthusiastic acceptance (Bartel *et al.*, 2001), it is largely based on unpublished results, which prevents its full evaluation. Moreover, currently available information does not provide sufficient grounds for accepting the claim that YUCCA directly participates in IAA biosynthesis. Of the three lines of evidence mentioned above, determination of YUCCA's protein function is the weakest. Functional assignment of an unknown protein is clearly not an easy task. In the best case scenario, substrates and products of the putative reaction are identified as endogenous components, *in vitro* activity is tested with an array of similar compounds to check its specificity and finally *in vivo* tests are performed. The only such information that has been gathered for YUCCA is the structure of an *in vitro* reaction product. However, if YUCCA does indeed catalyse the N-hydroxylation of tryptamine *in vivo*, the plants' apparent resistance to 5-methyl-Trp, which at least partially contradicts this functional assignment, must be explained. Resistance to toxic analogues is a very powerful tool for identifying proteins working directly on non-toxic variants of compounds. If used properly (see Hull *et al.*, 2000, for an excellent example), it can provide critical evidence for the involvement of a given protein in a studied pathway. The problem with this approach in the case of YUCCA is that the protein does not work directly on tryptophan. In order to be resistant, plants have to first convert a toxic analogue of Trp into (presumably non-toxic; see Goddij *et al.* 1993) 5-methyl-tryptamine, which may then be a substrate for YUCCA. Thus, YUCCA cannot be solely responsible for the resistance, and the rate-limiting step would be synthesis of tryptamine, catalysed by tryptophan decarboxylase (TDC). Further, if *YUCCA* overexpression is the cause of IAA overproduction, then TDC activity must be present in both wild-type and transgenic plants. Aside from the fact that such activity has never been reported in *Arabidopsis* (although putative aromatic amino acid decarboxylase genes have been found), wild-type plants should also be at least partially resistant to 5-methyl-Trp, and YUCCA protein overproduction could possibly enhance this resistance by progressive removal of tryptamine/5-methyl-tryptamine, thus increasing the flow of metabolites through the pathway. Still, the primary cause of toxic Trp analogue resistance would be TDC rather than YUCCA activity. This could be easily verified by monitoring the concentrations of 5-methyl-tryptamine and 5-methyl-IAA but, regrettably, no such experiments have been performed. It is therefore entirely possible that *YUCCA* overexpression exerts its effects through a completely different mechanism. It may, for example, accelerate Trp metabolism or affect synthesis of the other aromatic amino acids in a way that increases the concentration of Trp and/or its metabolites, which in turn makes the plants resistant to 5-methyl-Trp and induces IAA overproduction. To conclude, confirmation of YUCCA's direct involvement in IAA biosynthesis needs much more experimental work and in its current form this hypothesis is purely speculative. A recently proposed regulatory mechanism, in which reduced YUCCA activity leads to increased levels of tryptamine, thus inhibiting the CYP83B3 enzyme and diverting IAOx into the IAA biosynthesis pathway (Celenza, 2001), should probably also be reconsidered.

Conclusions and future prospects

This thesis describes the development and application of instrumental methods for analysing auxin metabolism in plants. These tools were designed to aid the investigation of metabolic pathways involving biosynthesis and catabolism of the primary auxin, indole-3-acetic acid (IAA), in *A. thaliana*. Based on the published results, work with this model plant can be summarised as follows:

- Exogenous IAA metabolism in *Arabidopsis* is similar to that previously observed in other dicotyledonous plants, and the major *in vivo* metabolites are amide conjugates and non-decarboxylative oxidation products.
- Depending on the concentration of exogenous IAA used in the experiment, either conjugation to aspartic and glutamic acid or direct non-decarboxylative oxidation may be the primary metabolic route.
- The indole part of some amide conjugates formed from exogenous IAA can be further oxidised, thus permanently deactivating them as a source of the IAA.
- Non-decarboxylative catabolism is the primary pathway of endogenous IAA deactivation.
- Endogenous steady-state levels of major amide conjugates in *Arabidopsis* are very low. The same can be said about the levels of amide conjugates that are potentially hydrolysable. This may indicate that they are used only for "fine-tuning" IAA homeostasis and that bulk changes of active hormone levels must occur through the transport, *de novo* synthesis or hydrolysis of high molecular weight conjugates.
- *Arabidopsis* contains an enzymatic activity that is essential for the synthesis of ester IAA conjugates, but no corresponding products other than the glucose ester have been identified so far.
- Mutations affecting the biosynthesis of indole glucosinolates in *Arabidopsis* can cause increased production of IAA and, thus, its major metabolites. However, such overproduction may represent an alternative routing of metabolites rather than the native biosynthetic pathway.
- Blocks in biosynthesis of the other types of glucosinolates can often result in increased levels of indole glucosinolates and consequently also auxins. This may be due to an increase in the intermediate pool shared by indole glucosinolates and the IAA.

Progress in understanding auxin metabolism in plants clearly cannot be achieved by the most popular approach of recent times (molecular genetics) alone. Development of more integrated and comprehensive methods of analysis is absolutely essential, as the results obtained from different fields of plant science need to corroborate each other. Research presented here focused on an attempt to build bridges between plant molecular genetics, analytical chemistry and plant biochemistry. Continuation of this work will hopefully lead to valuable answers and eventually further questions. In the short term, the major objective should be to improve the quantification methods for IAA metabolites and precursors, to allow dynamic pathway analysis with D_2O. This is an essential step, which should

lead to better understanding of relationships between different metabolites. At the same time, more efforts to identify both the metabolites and proteins involved are needed. In this respect, the regulation of IAA metabolism as well as the other growth regulators is still largely unknown. Without this knowledge, the mechanisms involved in plant hormone interactions - a fundamental aspect of plant physiology – may be very difficult to investigate and resolve.

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