Distribution and Regulation of Auxin in Arabidopsis Root Cells

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Cover: Mannitol, beautiful but annoying…
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Abstract

The plant hormone auxin (IAA) coordinates many of the important processes in plant development. For example, IAA is critical for normal embryogenesis, root development, cell elongation, and the tropic responses such as gravitropism and phototropism. IAA gradients are established and maintained in many tissues and it is thought that these gradients act as developmental cues, determining the fate of cells and tissues.

Descriptions of auxin distribution patterns with cellular resolution have previously been based solely on visual characterisation of auxin responsive reporter constructs. Measurements of the hormone gradient itself have been made in cambial sections of hybrid aspen and Pine, although even in this study, the measurements were not truly at cellular resolution.

In this thesis, I describe the measurement of IAA levels in the Arabidopsis root with cellular resolution. By combining the use of Arabidopsis lines expressing cell-specific GFP, Fluorescent Activated Flow Cytometry, and high-resolution mass spectrometry, I have provided the first analytical evidence for the presence of an IAA gradient in the Arabidopsis root apex. The work has enabled the development of an auxin distribution map for the root with cellular resolution.

The IAA content of cells is actively regulated in order to maintain the IAA gradient. This regulation has been shown to be achieved through a combination of active transport and *de novo* biosynthesis. It has been proposed that IAA catabolism and conjugation also contribute to the regulation of hormone levels.

In this thesis, I present data supporting the hypothesis that catabolism, by converting IAA into its major inactive catabolite oxIAA, contributes to the regulation of the levels of free IAA. I also present data showing a role for the plant hormone cytokinin in the dynamic regulation of IAA biosynthesis in response to changing developmental and environmental conditions.

Keywords: auxin, Arabidopsis thaliana, root, GFP, Fluorescent Activated Cell Sorting, mass spectrometry, single cell, catabolism, conjugation, oxIAA, Cytokinin.

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Till Mormor, nu har björkarna slagit ut…

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Petersson, S.V.**, Johansson, A.I., Kowalzcyk, M., Makoveychuk, A., Wang, J.Y., Moritz, T., Grebe, M., Benfey, P.N., Sandberg, G. and Ljung, K. (2009): An Auxin Gradient and Maximum in the Arabidopsis Root Apex Shown by High Resolution Cell Specific Analysis of IAA Distribution and Synthesis. *Plant Cell* 21(6), 1659-1668.

II **Petersson, S.V.**, Henyková, E., Simon, S., Greenham, K., Novák, O., Kowalzcyk, M., Estelle, M., Zazímalová, E., Sandberg, G. and Ljung, K. (2011): IAA catabolism - an important regulator of auxin homeostasis in *Arabidopsis*. (Manuscript).

III Jones, B.*, Gunnerås, S.A.*, **Petersson, S.V.***, Tarakowski, P., Graham, N., May, S., Dolezal, K., Sandberg, G. and Ljung, K. (2010): Cytokinin Regulation of Auxin Synthesis in Arabidopsis Involves a Homeostatic Feedback Loop Regulated via Auxin and Cytokinin Signal Transduction. *Plant Cell* 22(9), 2956-2969.

* To be considered joint first authors

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"Anyone who has never made a mistake has never tried anything new."
Albert Einstein

Abbreviations

All abbreviations are explained when they first appear in the text.

1 Introduction

Plant hormones are naturally occurring organic substances that at very low concentrations influence growth and development in a fundamental way. Plant hormones, or plant growth regulators as they are otherwise known, are defined differently to mammalian hormones. In mammals, hormones are defined as substances that are synthesized in a specific tissue or organ, are transported through the bloodstream to their target tissue, where they control physiological responses in a concentration dependent manner. By contrast, we now know that the synthesis of plant growth regulators is not restricted to specific organs to the same extent as mammalian hormones. Whereas they do move through vascular tissue (comparable to the blood stream), they also move through active cell-to-cell transport in a wide range of cell and tissue types. In addition, ethylene, which regulates a broad array of processes is a gas and moves by passive diffusion. Whereas the terms growth regulator and plant hormones can be used interchangeably, for simplicity, the term 'hormones' will be used hereafter in this thesis.

The hormone auxin, the main focus of this thesis, has a vast influence on plant development. The most common of the naturally occurring auxins is indole-3-acetic acid (IAA). Amongst a range of functions, auxins are known for stimulating cell elongation and root initiation; it is involved in apical dominance; mediates trophic responses to gravity and light and stimulates vascular tissue differentiation (Davies, 2004; Abel & Theologis, 2010)

Early work with auxin led it to being known as the root-forming hormone (Went, 1929). Synthetic auxins are now widely commercially available for the induction of root development in plant cuttings. Plant roots are responsible for nutrient and water uptake and as a result of being constantly subjected to changes in their soil environment are highly adaptable (Chapin, 1980). The root has to avoid or cope with toxic elements, microbes and herbivores and navigate around obstacles in the soil

as well as adapting to optimise the plant's ability to endure changes in nutrient and water availabilities. Root architecture changes by the production of lateral roots and root hairs, both of which increase root surface area and thus allow for an optimised uptake of nutrients and water. In Arabidopsis, root hairs can represent up to 70% of the total root surface area (Lòpez-Bucio et al., 2003). The plant hormone auxin plays key roles in the formation of both lateral roots and root hairs, it is also essential for the formation and activity of the root apical meristem and the stem cell niche (reviewed by Teale et al., 2005).

Similarly to mammalian hormones, specific auxin distribution patterns in the root provide developmental cues (Sabatini et al., 1999). The auxin molecule does not, however, result in conformational changes when it binds to the major receptor, TIR1 (Calderon-Villalobos et al., 2010). Rather, it acts as a molecular "glue" (Tan et al., 2007). Because auxin gradients have been detected in the root and other tissues, it has long been debated whether auxin can be seen as a morphogen. A morphogen is a compound that functions in a concentration dependent manner directly on target cells, rather than through signalling intermediates. Several classes of mammalian signalling molecules have been described as morphogens (Bhalerao & Bennett, 2003). Auxin has also been described as a morphogenic trigger where only a threshold level needs to be reached for it to activate a response (Benkova et al., 2009).

Whereas responses to auxin are partially regulated by differential ability of cells to respond to the hormone, plants also actively and constantly regulate cell and tissue levels of free auxin in order to have the appropriate concentrations, in the right tissues, at the right time. Ensuring appropriate auxin concentrations is achieved by an intricate coordination of IAA synthesis, degradation, conjugation, and active and passive transport. Importantly, IAA and the developmental programs it affect are also moderated by interactions with other hormones and the environmental stimuli.

1.1 Introduction to IAA Biosynthesis

Given the power of auxin to regulate plant physiology, it is not surprising that a wide variety of interconnected processes have evolved to regulate the levels of free IAA. One of the mechanisms by which it does this is by synthesising more IAA. Unfortunately, although it is clear that plants actively regulate the auxin biosynthetic rate, a number of auxin biosynthetic

pathways have been shown to co-exist and not all of them are fully characterised. In one case, that of the tryptophan independent pathway (Figure 1), there is only circumstantial evidence of its existence based on feeding studies (Wright et al., 1991). This does not mean, however, that the tryptophan independent pathway is insignificant. Work in tobacco protoplasts has shown that the tryptophan independent pathway plays a major role, as only 25% of the synthesized IAA originated from the known tryptophan dependent pathway (Sitbon et al., 2000). Furthermore, it appears that all pathways do not exist in all species of plants. The IAOx pathway for example (Figure 1), exists mainly in plants from the *Brassica* genus. The model plant, *Arabidopsis thaliana,* is a member of this family. As much of the work unraveling the auxin biosynthetic pathways are conducted in Arabidopsis this is an important consideration. One consequence of the lack of a complete picture of the pathways of IAA biosynthesis is that the interpretation of analytical data is always done in the light of the most recent model of IAA biosynthesis and as new information on the biosynthetic pathways becomes available the data may need to be re-evaluated and interpreted.

Tryptophan is the precursor for the auxin synthesized by all the IAA biosynthesis pathways described in the following section. Tryptophan is also the precursor for the camalexin and indole glucosinolate biosynthesis pathways and, therefore, as tryptophan can be limiting, IAA biosynthesis has to compete with these pathways (Normanly, 2010). In humans, tryptophan is a precursor for the neurotransmitter serotonin, the hormone melatonin and vitamin B_3. Melatonin and serotonin have also been found in plants. A study by Murch et al. (2002) showed that an increase in the endogenous concentration of melatonin was correlated with an increase in de novo root formation, and increased serotonin levels corresponded to increased shoot formation in St. John's wort (*Hypericum perforatum*) plants. The IAA biosynthesis pathways described in this thesis are depicted in Figure 1.

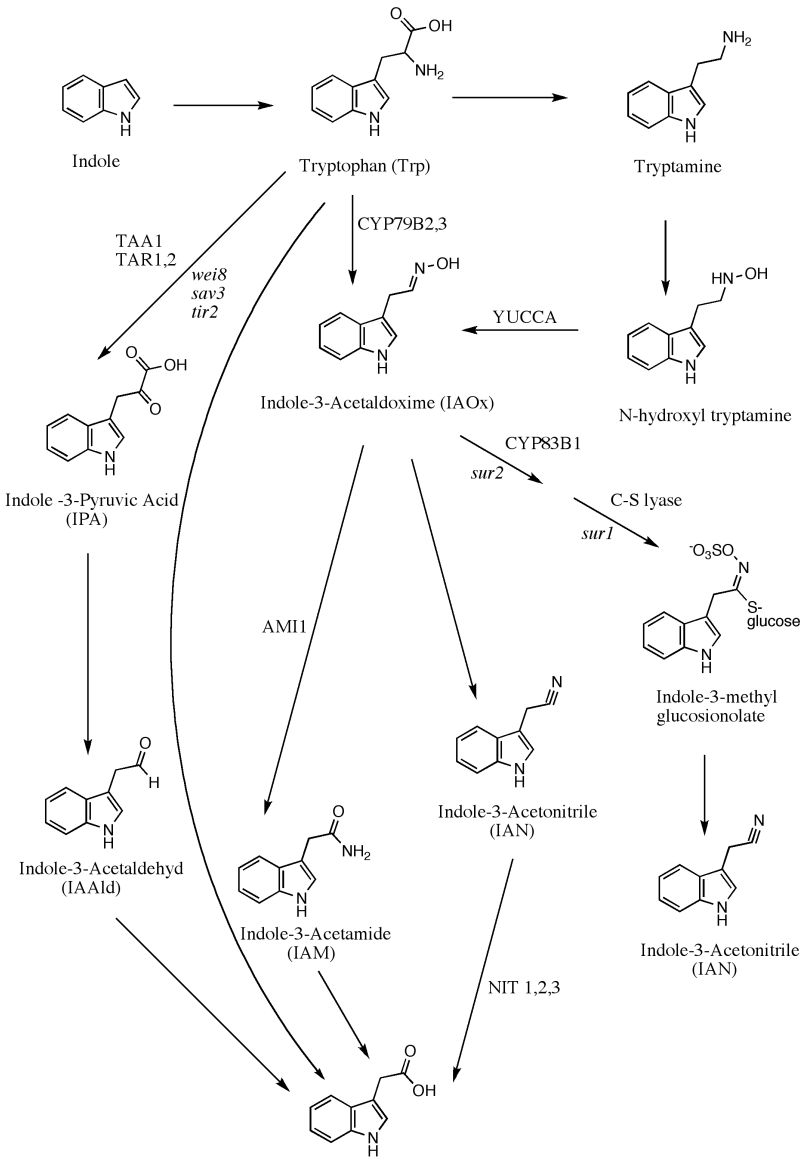

Figure 1. The tryptophan dependent IAA and indole-glucosinolate biosynthesis pathways. Gene name is written in capital letters along arrows, and mutants in italics.

1.1.1 The YUCCA Pathway

It has been proposed by Zhao et al. (2001) that in the YUCCA biosynthetic pathway (Figure 1), tryptamine is converted to N-hydroxytryptamine by

members of the YUCCA enzyme family and is then converted to IAOx by an as yet unidentified enzyme. *YUCCA* homologs have been found in all sequenced plant genomes and therefore seem to be highly conserved in the plant kingdom (Zhao, 2010). Tryptamine has also been shown to be converted directly to IAA through IAAld in pea (*Pisum sativum*) (Quittenden, 2009).

YUCCAs (i.e. *YUC1*) encode flavin monooxygenase-like enzymes that are believed to catalyze the hydroxylation of the amino group of tryptamine, which is thought to be a rate-limiting step in tryptophan dependent auxin biosynthesis of many species. The dominant *yuc1* mutant has increased levels of IAA compared to WT (Zhao et al., 2001) and has a phenotype typical of elevated IAA levels, with long hypocotyls, epinastic cotyledons and elongated petioles when grown in white light. *yuc1* roots are shorter than wild-type (WT) roots, but the root hairs are longer and more plentiful than those of WT seedlings (Zhao et al., 2001). In Arabidopsis, the *YUCCA* family is comprised of 12 genes (Cheng et al., 2006). Several of them appear to have redundant functions (Cheng et al., 2006). Recently, the Zhao et al. (2001) data was reexamined and after extensive MS-MS analysis it was found that N-hydroxytryptamine might not be the product of tryptamine metabolism by the YUCCA enzymes after all (Quittenden, 2009). This suggests that the role of YUCCA in IAA biosynthesis may not be as crucial as previously thought. However, as the quadruple *yuc1 yuc4 yuc10 yuc11* mutant is not able to develop a hypocotyl and a root meristem; and the triple mutant *yuc1 yuc4 pin1* fails to form leaves and flowers (Cheng et al., 2007) the YUCCA protein clearly play an important role in plant development. It remains possible that the YUCCAs are involved in the synthesis of other, auxin like compounds (Tivendale et al., 2010).

1.1.2 The IAOx Pathways

The route for conversion of IAOx to IAA was first successfully addressed in a feeding study where $^{13}C_6$ labelled IAOx was fed to Arabidopsis mutants deficient in the cytochrome P450 enzyme, CYP79B. Instead of being channelled to the production of indole-glucosinolates, two other labelled intermediates, indole-3-acetamide (IAM) and indole-3-acetonitril (IAN), were found. IAM, IAAld and IAN are assumed to be precursors of IAA in the IAOx pathway. IAN is converted by the Nitrilase 1,2 and 3 genes to IAA (Sugawara et al., 2009).

The cytochrome P450 enzyme family members, CYP79B2 and CYP79B3 metabolize tryptophan (Trp) to Indole-3-acetadoxime (IAOx) *in*

vitro and when the genes are over-expressed in Arabidopsis they give typical IAA overproduction phenotypes, with long hypocotyls and epinastic cotyledons (Zhao et al., 2002). It is likely that the over production of IAA is a result of an increased flux of IAOx into IAA biosynthesis (Hull et al., 2000; Zhao et al., 2002). To date, *CYP79B2/B3*-like genes have only been identified in Brassicas (Zhao, 2010). The IAOx pathway was further defined in Arabidopsis when two auxin overproducing mutants, *sur1* (Boerjan et al., 1995) and *sur2* (Hull et al., 2000; Zhao et al., 2002) were characterised.

In the recessive *sur1* mutant there is an increased abundance of both free and conjugated IAA, and the roots do not develop normally, as connections between the stele, cortical and epidermal cells disintegrate. Other severe phenotypes include an excess of adventitious and lateral roots, a reduced number of leaves, and the absence of an inflorescence (Boerjan et al., 1995). SUR1 was later found to be a C-S lyase integral to the glucosinolate biosynthetic pathway. In the *sur1* mutant, glucosinolates do not accumulate to the same extent as in WT as IAOx is redirected from the glucosinolate pathway towards IAA (Mikkelsen et al., 2004).

SUR2 similarly controls a key point in the regulation of IAA biosynthesis (Delarue et al., 1998). SUR2 encodes the stress-induced cytochrome P450, CYP83B1. CYP83B1 catalyses the first step in the synthesis of indole glucosinolates from IAOx. As a result of the mutation of the *SUR2* gene, the *sur2* mutant also has elevated concentrations of IAA (Barlier et al., 2000). *sur2* cotyledons are small and epinastic and true leaves rarely form. The hypocotyl epidermis disintegrates as many sub-epidermal adventitious root primordia form. However, most of the root primordia remain quiescent (Delarue et al., 1998). The IAOx pathway is proposed to be specific to indole glucosinolate producing species, although nitrilases have been isolated from maize that are thought to be capable of converting of IAN to IAA (Normanly, 2010).

1.1.3 The IPA Pathway

The IPA pathway (Figure 1) has been shown to be one of the major tryptophan dependent IAA producing pathways. In this pathway, Trp is converted to IPA, which is then converted to IAA aldehyde, which is finally oxidised to IAA. The only genes that have been characterized in this pathway are *TRYPTOPHAN AMINOTRANSFERASE* (*TAA1*) and its homologs *TRYPTOPHAN AMINOTRANSFERASE RELATED 1-4* (*TAR1-4*) (Yamada et al., 2009, Tao et al., 2008, Stepanova et al., 2008). The encoded proteins are localised in the cytoplasm (Yamada et al., 2009).

TAA1 is an aminotransferase that catalyses the formation of IPA from L-tryptophan (Yamada et al., 2009). *TAA1* mutants have phenotypes indicating a perturbation of IAA synthesis. For example the *TAA1* mutant, *sav3,* has shorter hypocotyls than the WT when grown in conditions that would normally induce a shade avoidance response (Tao et al., 2008). TAA1 has been shown to be involved in rapid auxin biosynthesis required for mediating shade response morphological adaptations. Tao et al. (2008) showed that when WT seedlings are moved into simulated shade conditions IAA levels rapidly increase. In contrast, TAA1 deficient seedlings show decreasing IAA levels under the same conditions (Tao et al., 2008). Another mutant allele of *TAA1*, *weak ethylene insensitive8* (*wei8*), was isolated on the basis of an ethylene response phenotype, indicating that the TAA1 gene is involved in mediating processes involving both auxin and ethylene (Stepanova et al., 2008).

The *TIR2* gene has also been proposed to be involved in the IPA pathway and is identical to TAA1 gene, encoding a tryptophan aminotransferase (Yamada, et al., 2009). The *tir2* mutant has a short hypocotyl phenotype that can be rescued by the addition of exogenous IAA. *TIR2* mRNA accumulate in response to high temperatures and preferentially on the lower side of roots responding to gravity, indicating an involvement in high temperature and gravitropic responses (Yamada et al., 2009, Stepanova et al., 2008).

1.1.4 The IAM pathway – Does it Exist in Plants?

Some plant pathogens can convert tryptophan to indole-3-acetamide (IAM) by the tryptophan-2-monooxygenase iaaM and subsequently hydrolyse it to IAA by iaaH. It is the only completely characterised IAA pathway (Figure 1), and although IAM has been detected in plants (Sugawara, 2009) it has been postulated that plants generally do not use this pathway (Zhao, 2010). Genes from the AMIDASE 1 (AMI1) family encode indole-3-acetamide hydrolases that have been found to convert IAM to IAA (Mano et al., 2010). AMI genes have been isolated from Arabidopsis (AtAMI1) and *Nicotiana tabacum* (NtAMI1) (Mano et al., 2010). In the face of accumulating evidence it is possible that the supposition that the IAM pathway is not important in plants may have to be abandoned.

1.2 IAA Catabolism – A Way for the Plant to Maintain IAA Homeostasis

Just as *de novo* biosynthesis is an important mechanism for ensuring appropriate levels of IAA are maintained, so too is the catabolism of the hormone (Normanly, 1997). IAA catabolism can be divided into oxidation and conjugation, although the two mechanisms are strongly intertwined (Figure 2).

1.2.1 Non-Decarboxylative Oxidation of IAA

The major route for IAA catabolism is non-decarboxylative oxidation (figure 2). In this pathway, the indole ring of free IAA is oxidised to the major catabolite, oxoindole-3-acetic acid (oxIAA) (Östin et al., 1998). IAA-amino acid-conjugates can also undergo oxidation. For example, IAAsp can be oxidized to oxIAAsp in tomato pericarp (Östin et al., 1995; Östin et al., 1998). In maize, oxIAA can be further catabolised to 7-OH-oxIAA-Glc (Nonhebel et al., 1985). In *Citrus sinensis* IAA is catabolised predominantly by one of two ways; by converting IAA into DioxIAGlc via oxIAA or by converting IAA into oxIAAsp via IAAsp (Chamarro et al., 2001). In Arabidopsis, oxIAA-Glc can be formed both from oxIAA and IAGlu (Kai et al., 2007). These examples indicate how tightly linked non-decarboxylative oxidation of IAA, and IAA conjugation are to each other.

1.2.2 IAA Decarboxylation

Another potential pathway of IAA catabolism is through decarboxylation. Little is known about the process of IAA decarboxylation, where plant peroxidases modify both the indole-ring and the side chain. The main products of IAA decarboxylation are indole-3-methanol, indole-3-aldehyde, indole-3-carboxylic acid, 3-hydroxymethyloxindole, and 3-methyleneoxindole (Bandurski et al., 1995). *In vitro* experiments with lupine roots showed that indole-3-methanol is the major IAA oxidation product in this species (Ros Barceló et al., 1990). Kerk et al. (2000) found that in radish, oxidative decarboxylation of IAA occurred mainly in the root apex. Over-expressing peroxidases in tobacco do not alter the IAA levels, although the same peroxidases can degrade IAA *in vitro* (Lagrimini et al., 1997).

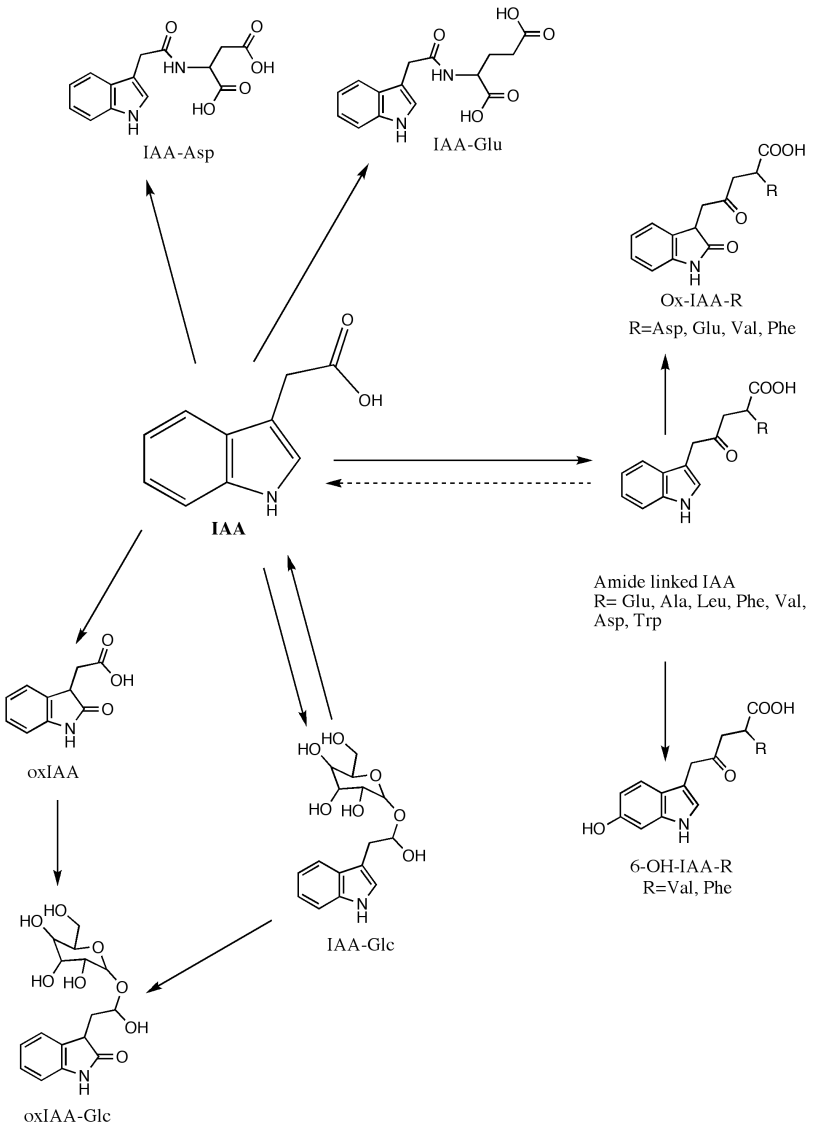

Figure2. Major routes for IAA catabolism in Arabidopsis; conjugation to sugars and amino acids and non-decarboxylative oxidation.

1.3 IAA Conjugation

Conjugation leads to a change in the IAA side chain (figure 2). For some conjugates, this is a reversible process and the conjugates can be hydrolysed back to active IAA. In seeds, the conjugated form of the hormone is the

predominant form of IAA and rapid hydrolysis of the conjugated IAA during germination releases free IAA to the growing shoot and root, which are not able to produce *de novo* IAA during the early stages of growth (Bandurski et al., 1995). There are two types of conjugation; the ester-type conjugates in which IAA is linked to a sugar or cyclic poly-ols, and amide-type conjugates where IAA is bound to amino acids or poly-peptides (Ljung et al., 2002). The amide linked conjugates IALeu, IAPhe and IAAla can be hydrolysed back to free IAA (Bartel & Fink, 1995; Davies 1999).

The first protein-IAA conjugates were found in bean (*Phaseolus vulgaris*). IAA protein conjugates accumulate during seed maturation and decline during germination, suggesting a role in the accumulation of storage compounds (Bialek and Cohen, 1986, Bialek and Cohen, 1989). Since their initial identification, protein-IAA conjugates have been identified in a number of species, and it has been shown that the type of conjugates depends on the species in question (Bandurski et al., 1995).

The conjugates that have been found in Arabidopsis, the model plant used in this study, are IAAsp, IAGlu, oxIAA-hexose (Östin et al., 1998) IAA-Gln (Barratt et al., 1999) IAGlc, IAGlu and IAAsp (Tam et al., 2000) IAAla and IALeu (Kowalczyk & Sandberg, 2001) 6-OH-IAA-Phe, 6-OH-IAAVal and oxIAA-Glc (Kai et al., 2007) (figure 2). The major IAA catabolite is oxIAA, and the most abundant conjugates in Arabidopsis are IAAsp and IAGlu, which cannot be hydrolysed back to active IAA (Östin et al., 1998). In Arabidopsis, the majority of all conjugation therefore appears to be an irreversible process acting solely to reduce IAA levels. When Arabidopsis plants are fed with low IAA concentrations (below 0,5μM) most of the exogenous hormone is oxidised to oxIAA. In contrast, when high concentrations (above 5μM) of IAA are applied metabolism is instead redirected towards IAA conjugation, resulting in the formation of mainly IAAsp and IAAglu (Östin et al., 1998). This relationship between IAA oxidation and conjugation was reported as early as 1992, when tomato pericarp was fed with high IAA concentrations (Riov & Bangerth, 1992). This work showed a concomitant increase in the formation of IAAsp and a decrease in the formation of the conjugate, oxIAA (Riov & Bangerth, 1992). Increasing concentrations of exogenous IAA push metabolism towards conjugation instead of oxidation. This corresponds well to the low levels of IAA conjugates seen in WT under normal conditions, indicating that the oxIAA pathway is sufficient for IAA regulation by catabolism (Ljung et al., 2002).

A few of the enzymes involved in conjugation and hydrolysis of conjugates have been isolated. The *IAA LEUCINE RESISTANT1* (*ILR1*)

gene, an IAA-conjugate hydrolase, has strong specificity towards IALeu and IAPhe in Arabidopsis (Bartel & Fink, 1995). *IAA ALANINE RESISTANT 3* (*IAR3*), an ILR1 homolog, has been shown to be an IAAla hydrolase (Davies et al., 1999). An *ilr1 iar3* double mutant was less sensitive than WT to many of the conjugates, indicating that IAR3 and ILR1 are involved in the hydrolysation of the conjugates *in vivo* (LeClere et al., 2002). The *GH3* gene family encodes IAA-amide synthases in Arabidopsis that catalyse the formation of amino acids - IAA conjugates. *In vitro* assays showed that Ala, Asp, Phe and Trp conjugates could be formed by GH3 (Staswick et al., 2005). This was subsequently verified by GC-MS. Further verification by TLC indicated that Glu, Gly, Gln, Tyr, Ile and Val conjugates could also be formed through the action of GH3 (Staswick et al., 2005). Early work suggested that some of the IAA conjugates themselves have IAA activity, but those conjugates were shown to be the ones that could be hydrolysed back to free IAA. A likely explanation for that observation was that the conjugates themselves did not exert an IAA effect, but rather the conjugate was hydrolysed and it was the IAA released that caused the effect (Hangarter et al., 1980). *In vitro* feeding studies showed that IAPhe and IAVal could act as precursors for the oxidative IAA metabolites: 6-OH-IAA-Phe and 6-OH-IAA-Val. In the plant 6-OH-IAA-Phe and 6-OH-IAA-Val are only present in very low levels (Kai et al., 2007).

1.4 IAA Transport in the Plant

Working alongside this *in situ de novo* IAA synthesis and conjugation, active transport of the hormone functions to achieve cell and tissue concentration maxima of the hormone. IAA transport is essential for regulating the distribution of IAA and for building up IAA concentration gradients within tissues. IAA can be transported symplastically in the phloem together with other signalling compounds and photoassimilates to sink tissues. Given that auxin moves in tissues other than the vasculature, it was suggested early on that IAA moves symplastically in other tissues via the plasmodesmata. However, in plasmolysed wheat coleoptiles, where most of the plasmodesmatal connections have been lost, polar IAA transport still occurred, suggesting that other routes of transport are available (Cande & Ray, 1976). The conclusion was that IAA most likely moves from cell to cell by crossing the plasmalemmas and the apoplast, rather than via plasmodesmata. It was subsequently shown that this apoplastic/symplastic

movement is the main form of movement in the polar auxin transport stream (Cambridge & Morris, 1996).

1.4.1 Polar Auxin Transport and the Chemiosmotic Hypothesis

Polar IAA transport is a combination of diffusion and active efflux and influx. IAA is a weak acid (pk_a=4.85) and its ability to cross the plasma membrane therefore depends on the pH. The pH in the cytoplasm is around pH=7, which means that intracellular IAA exists mainly in its dissociated form, IAA^-+H^+. In the apoplast the pH is around pH5.5, and IAA exists mainly in its non-dissociated form, IAAH. The chemiosmotic hypothesis states that the proton motive force across the membrane provides the driving force for IAA transport (Rubery & Sheldrake, 1973; Raven, 1975). This means that IAA can enter the cell either by diffusion in a non-energy dependent way or by an uptake symporter. By contrast, IAA has to be actively transported out of the cell by an anion efflux carrier (Lomax et al., 1995). In the apoplast, IAA can diffuse as far as 10 um before entering the next cell. Different cell walls have different permeability for small organic acids like IAA. The diffusion rate in mature epidermis cell walls is for example 10-fold slower compared to the mature cortex (Kramer, 2007).

1.4.2 IAA Efflux - The PIN and ABCB/PGP Proteins

Polar auxin efflux from cells is conducted by the membrane-bound PIN (PIN FORMED) protein family members. Synchronised asymmetric intracellular localisation of PIN proteins means that auxin flow is directed sequentially through cells and tissues to regions where it is required for action. Eight members of the PIN family have been identified in Arabidopsis and they can be divided into two groups: (I) PIN1, 2, 3, 4, 7 and 8 are located in the plasma membrane; and (II) PIN 5 and 6 are located in the endomembrane (Benjamins & Scheres, 2008, Zazimalova et al., 2010).

PIN's located in the plasma membrane

Arabidopsis *PIN1* was the first PIN protein to be identified. The *pin1* mutant was first described in 1991 by Okada et al. these authors suggested that it might be involved in IAA transport as the phenotype resembled a plant grown on an IAA transport inhibitor (Okada et al., 1991). The *pin1* mutant has a naked pin-formed inflorescence with either only a few defective flowers or no flowers at all. The PIN1 auxin efflux carrier is

located at the lower side of cells in vascular tissue, consistent with the direction of IAA transport (Gälweiler et al., 1998). Similarly, the PIN2 protein is polarly localised to the plasma membranes of epidermal and cortical cells in the elongation and meristematic region of the root. The *pin2* mutant is agravitropic (Müller et al., 1998). The PIN3 protein is normally symmetrically localized in columella cells, the gravity-sensing tissue in roots. PIN3 localization changes rapidly after reorientation of the roots to provide a change in the gravistimulation vector. It has been observed that PIN3 re-localization has been observed in as little as 2 min after reorienting the roots and that the process of re-localization can be complete after only 5 min. This rapid re-localization allows the plant to quickly redirect IAA flow and growth in order to react to the altered gravistimulation (Friml et al., 2002b). PIN4 is polarly localized in root meristems where it helps to stabilize the IAA maxima in the quiescent center and stem cells (Friml et al., 2002a). *pin4* mutants can neither establish nor maintain endogenous auxin gradients in developing and mature root meristems. In this mutant the well-defined quiescent center is replaced by cells that have divided irregularly (Friml et al., 2002a). PIN7, together with PIN1 and PIN4 is important for determining auxin gradients and apical–basal axis establishment during early embryogenesis (Friml et al., 2003).

PINs located in the ER membrane

PIN5 is required for normal root initiation and root and hypocotyl growth. However, unlike the other PINs it does not transport IAA from cell-to-cell but is instead located in the ER membrane. It has been suggested that it regulates IAA transport by reducing the amount IAA available for efflux out of cells via the plasma membrane-bound PIN proteins. Inducing the PIN5 expression in tobacco cells resulted in a pronounced decrease in free IAA levels and an increased capacity to produce the amino acid conjugates IAAsp or IAGlu. These data suggest that the role of PIN5 is very different from the other characterised PIN proteins (Mravec et al., 2009). The function of PIN6 and PIN8 have not yet been identified, however, PIN8 has been shown to be located both in the plasma membrane and the ER (Ganguly et al., 2010)

ABCB/PGP transporters

The ABCB/PGP P-glycoproteins are members of the ATP-binding cassette (ABC) protein family that transport hormones, lipids, metals, secondary metabolites and xenobiotics (Verrier et al., 2008). In Arabidopsis, ABCB proteins are localized non-polarly in the plasma membrane (Zazimalova et

al., 2010) and maintain long-distance auxin transport streams and movement of auxin out of apical tissues (Blakeslee et al., 2007). ABCB has the ability to stabilize PIN proteins and enhance auxin transport at certain developmental stages (Titapiwantanakun, 2009).

1.4.3 Active IAA Influx

There are four known putative auxin influx carriers in the Arabidopsis genome, the AUXIN RESISTANT1 (AUX1) and the Like AUX1 proteins; LAX1, LAX2, and LAX3 (Parry et al., 2001). *AUX1* is expressed in the root apex. The *aux1* mutant shows agravitropic growth and is resistant to the membrane impermeable synthetic auxin 2,4-D, indicating a role in auxin uptake (Bennett et al., 1996). AUX1 is asymmetrically localized to the plasma membrane of lateral root cap, columella and in root protophloem cells. AUX1 has also been shown to be involved in the unloading of IAA from the phloem (Swarup et al., 2001). LAX3 is a high affinity influx carrier expressed in mature tissues next to developing lateral root primordia. Its expression is auxin inducible. Auxin-dependent induction of cell-wall-remodeling enzymes is increased by LAX3 activity, promoting cell separation prior to lateral root emergence (Swarup et al., 2008).

1.5 Crosstalk Between IAA and Other Hormones

Most plant hormones are involved in root growth and development in some way, but IAA seems to have a central role in this complex networked process. A brief summary of the interactions of IAA with other hormones is presented below, with particular attention paid to auxin – cytokinin interactions and root growth and development.

Ethylene is known to stimulate the synthesis of IAA in the root apex (Swarup et al., 2007). During root growth, ethylene inhibits root cell expansion. Swarup et al. (2007) showed that ethylene up-regulates auxin biosynthesis to enhance its ability to inhibit root growth. Ruzicka et al. (2007) further showed that ethylene stimulates basipetal auxin transport and activates the auxin-signaling pathway in the elongation zone, leading to regulation of root growth by an inhibition of cell elongation. Up-regulation of the WEAK ETHYLENE INSENSITIVE genes WEI2 and WEI7 by ethylene triggers accelerated production of Trp and, ultimately, auxin biosynthesis in Arabidopsis roots. WEI2 and WEI7 encode the α and β subunits of anthranilate synthase, a rate-limiting enzyme in the Trp

biosynthesis (Stepanova et al., 2005). WEI2 and WEI7 are required for a high auxin biosynthetic rate in auxin-overproducing mutants, indicating a broader regulatory role for the WEI2 and WEI7 genes in auxin production (Stepanova et al., 2005). In contrast to these findings, Ross and coworkers recently (2011) failed to find any evidence supporting an up-regulation of IAA by ethylene, although these experiment were done in a different model system, namely pea (Ross et al., 2011).

Brassinosteroids (BR) are known to be involved in a number of physiological processes. For example, seed germination, stem and root elongation, vascular differentiation, cell expansion and apical dominance (Halliday et al., 2004). Several of these processes are known to be also regulated by IAA, which would imply an interaction at some level between the two hormones. A large number of genes are regulated by both IAA and BR in similar ways and BR regulates the expression of PIN and NIT3 genes that are involved in IAA transport and IAA biosynthesis (Halliday et al., 2004, Nemhauser et al., 2004).

Gibberellic Acid (GA) is mainly known as a regulator of seed germination and internodal cell elongation. IAA and GA crosstalk is also known to be important for cell expansion and tissue differentiation in the root. GA promotes the growth of plants by reversing the effects of growth repressor proteins called the DELLAs (Benkova & Hejatko, 2009). Auxin is necessary for GA-mediated degradation of the DELLA proteins in the control of root growth. Weakening of auxin transport or signaling in the plant delays the effect of GA-mediated DELLA protein destabilization, which is necessary for growth (Fu & Harberd, 2003). IAA has also been shown to increase transcript accumulation for several of the *GA20ox* genes involved in gibberellin biosynthesis (Frigerio et al., 2006).

Abscissic acid (ABA). The mode of IAA – ABA crosstalk in the root is not as well described as for some of the other hormone interactions. It is known, however, that ABA is involved in lateral root formation, a process highly dependent on IAA (De Smet et al., 2006b). The role of IAA in lateral root emergence is well known (De Smet et al., 2006a). ABA can inhibit IAA signaling and at the same time positively regulate IAA transport during lateral root emergence (De Smet et al., 2006a). The NO HYDROPHOBIC RESPONSE1 (NHR1) protein affects root meristem formation by regulating QC, columella stem cell and root cap cell development. It has been hypothesized that NHR1 is involved in an ABA-dependent IAA redistribution (Eapen et al., 2003).

1.5.1 IAA – Cytokinin Interactions

The interactions between auxin (IAA) and cytokinin (CK) were initially discovered when it was shown that plants could be regenerated from callus grown on agar supplemented with both IAA and CK (Skoog & Miller, 1957). In callus tissue, high levels of cytokinins promote shoot formation and high levels of IAA induce root formation. Equal IAA and CK concentrations tended to give rise to disordered growth in the callus tissue. The traditional view of the IAA – CK relationship was that IAA was produced in the shoot and transported to the roots and CKs were produced in the root and transported to the shoot (Cline et al., 1991). However, this simplistic model was shown to be incomplete. In the well-studied phenomenon of apical dominance, the presence of the shoot apical meristem inhibits outgrowth of axillary buds. This process was shown to be controlled, at least in part by crosstalk between IAA and CK (Cline et al., 1991). Auxin induces growth at the apex and cytokinins control and promotes the outgrowth of axiliary buds (Skoog & Miller, 1957). In pea, Tanaka et al. (2006) showed that IAA negatively regulates CK biosynthesis in the shoot apex by controlling the expression of phosphate-isopentenyltransferase (IPT) genes after decapitation. Removing the shoot apex, a primary source of IAA, promotes IPT expression and CK in the axillary meristems and axillary bud outgrowth.

The CK biosynthesis pathway

There are two types of CK, the isoprenoid and the aromatic types. The isoprenoid types such as N6-(Δ2-isopentenyl) adenine (iP), trans zeatin (tZ), and dihydozeatin (DZ), that come from the methylerythritol phosphate pathway (MEP) are the most common in plants. Another abundant CK is cis zeatin (cZ) that is synthesised from the mevalonate pathway (MEV)(Sakakibara, 2006).

Adenosine phosphate-isopentenyltransferase (IPT) catalyses the first reaction in the biosynthesis of isoprene cytokinins. The IPT genes were identified by Kakimoto (2001). The next step is conversion by the CYP735A genes to iPR, the precursor for the active cytokinin iP. The CYP735A1 CYP735A2 enzymes were identified by Takei (2004). The discoveries of the LONELY GUY (LOG) genes in rice were published by Kurakawa et al. (2007) The LOG genes encode cytokinin activating enzymes, which have cytokinin-specific phosphoribohydrolase activity. LOG proteins are able to perform the final step in cytokinin biosynthesis by converting the inactive cytokinin nucleotides to the active free base form (Kurakawa et al., 2007). Together with auxin, cytokinins are crucial players

in the regulation of plant growth and development. Like auxin, CK levels can be regulated by the activity of hormone degrading enzymes. For cytokinins, the major family of degrading enzymes are the cytokinin oxidases (CKX) (Schmülling et al., 2003).

Constitutive overproduction of CK and IAA through the overexpression of both *A. tumefaciens IPT* and iaaM or iaaH genes resulted in reduced levels of IAA and CK in 11 week old tobacco plants. Interestingly, the overall metabolite pattern in the two biosynthesis pathways were not altered (Eklöf et al., 1997). This shows that an alternative steady state can be reached in these plants and that IAA and CK respond to changes in the concentration levels of each other. In 2004, it was shown by Nordström et al. that feeding auxin to three week old Arabidopsis plants led to a decrease in the CK biosynthesis rate in a dose dependent manner after 24h at the whole plant level. Conversely, when CK levels were increased in an inducible system, IAA synthesis did not begin to decline until after 24-36 h. Because of the delayed response both by feeding IAA and inducing CK synthesis, it was thought that the regulation was indirect rather than being a direct control of CK and IAA by the other hormone. Importantly, in this work, the biosynthesis of CK was found not to be restricted only to the root system as previously thought. High rates of CK biosynthesis occurred in both the roots and shoots, and the increased biosynthesis occurred preferentially in young, actively dividing tissues.

1.5.2 Future Challenges for Analysing Auxin in Plant Material

IAA is mainly produced in young actively growing tissues (Ljung et al., 2001). The synthesis rates of the hormone are dynamic, changing with the developmental program and reacting to changes in the environment. Up until now it has not been possible to analytically quantify IAA levels on a cellular level, and a visual estimate with reporter genes have been used instead. For a comprehensive understanding of auxin levels and changes in levels it is crucial to understand how to best quantify the IAA levels, how and when to harvest which tissue, and how to extract the metabolites. As described here, auxin biosynthesis degradation, conjugation and transport all affect the steady state levels of the hormone.

To what extent should the IAA precursors and conjugates be included in the analysis, to get a more comprehensive picture? Is it necessary to quantify other hormones, as the hormones affect each other? Measuring only IAA may not be enough. Should different inhibitors of, for example, transport and protein synthesis be used in the experiments and if so how should one account for the other perhaps unknown effects of the inhibitor that is not

directly related to auxin but in turn will affect auxin? The more detail with which one examines IAA distribution the more important questions like these become.

2 Objectives

The aim of my thesis work was:

To develop methods enabling analysis of signalling molecules, metabolites and proteins in specific cell types of the Arabidopsis root.

Technologies for cell specific analysis of hormones, metabolites and proteins will be essential for future progress in the field of plant development. My aim was to develop methods to analyse these compounds with cellular resolution, utilising highly sensitive and selective mass spectrometry-based methods.

To utilise the developed methods to analyse auxin distribution in specific cell types of the root apex.

Auxin has an essential role in root development, controlling cell division and cell elongation, as well as cell fate determination. It is assumed that the auxin molecule exerts is action via concentration differences in finely tuned gradients. Only indirect evidence has been available for the existence of cell specific concentration differences of this hormone in the root apex. My aim was to present direct evidence for such a concentration gradient.

To investigate how the IAA gradient is regulated in the root, with focus on IAA catabolism and auxin – cytokinin interaction.

The mechanisms of controlling auxin gradients must involve enzymatic inactivation of the IAA molecule and/or a physical separation of IAA from its target cells. The aim was to describe how inactivation of auxin occurs in

Arabidopsis, thereby identifying potential mechanisms affecting auxin gradients.

Auxin – cytokinin interactions are one of the classical mechanisms controlling morphogenesis in plants. The aim was to investigate how cytokinin affects IAA levels, and how the crosstalk between IAA and cytokinin regulates IAA homeostasis, using the Arabidopsis root as a model system.

3 Methodological Overview

3.1 The Arabidopsis thaliana Root as a Model System

Arabidopsis has a wealth of advantages as a model plant. It is a small and fast growing plant with a lifecycle from seed to seed of as short as 2 months. It has a comparatively small genome (157 Mbp) that has been fully sequenced. As it is has been the most commonly used species for basic plant research for the last 30 years there are abundant, readily available genetic and data resources.

The simple cellular structure of the Arabidopsis root makes it a good system for studying root development (figure 3a). The root can be divided into the meristematic zone (extending approximately 250 μm from the apex), the elongation zone (approximately 250 μm long) and the differentiation zone, which spans the distance up to the formation of lateral root hairs (The GFP expressing cells that were isolated for FACS (Fluorescence Activated Cell Sorting) in the studies presented here originated predominantly from the meristematic and elongation zone). The organisation and number of cells are remarkably constant between roots. Each cell type constitutes a layer that is only one to a few cell layers thick. All cell types originate from the initials surrounding the quiescent centre and are arranged in well-defined cell files. The initials form three tiers; the protoderm (that forms the initials, root cap and endodermis), the periderm (that forms the cortex and endodermis) and the plerome (that forms the stele). The organisation of these cell files is highly conserved (Dolan et al., 1993). The structural consistency between individual Arabidopsis roots and its simplicity makes it a highly suitable model system.

3.2 The Green Fluorescent Protein (GFP)

The cloning of the Green Fluorescent Protein (GFP) from jellyfish (*Aequorea victoria*) revolutionised the study of proteins in biology. The use of GFP allowed researchers for the first time to easily track protein dynamics *in vivo*. So important was the discovery, that two of the researchers involved in the development of the GFP system for use in biology research were awarded the 2008 Nobel Prize for chemistry. The GFP protein is a relatively small protein and when fused to a protein of interest is usually expected to not interfere with the function of the tagged protein. In addition, GFP is generally not toxic to living cells (Chalfie et al., 1994).

Many of the GFP lines used for cell sorting in this study were from a GAL4-GFP enhancer trap collection of lines developed by Professor Jim Haseloff (University of Cambridge), others were kind gifts from Professors Ben Scheres (University of Utrecht) and Klaus Palme (University of Freiburg). The lines used in the study can be ordered from www.arabidopsis.info.

All of the lines used in the study express GFP in defined cell types. One potential drawback with fluorescent proteins in plants is that the auto-fluorescence emitted by plastids and cell walls can interfere with or confound the analysis of the signal generated by the introduced fluorescent protein. Additional problems can be caused by auto-fluorescence of cells damaged by high-energy laser excitation (DeBlasio et al., 2010). One of the benefits of using the primary Arabidopsis root, as the experimental system is that background auto fluorescence is normally minimal in this tissue.

The collection of GAL4-GFP lines that were finally chosen for the work presented in Paper I were in many ways not ideal, consisting, for example, of lines from two different ecotypes (Columbia and C24), but the collection of lines was the best available at the time. Some examples of the lines used here are schematically shown in figure 3b. An optimal collection of lines would have high intensity GFP signalling in non-overlapping cell types. As the FACS cell sorter used in the studies is able to sort cells with several different coloured fluorophores from a mix of cells, it would also be useful to have lines expressing different coloured fluorophores in separate cell types in a single plant line, particularly for lines with fluorophores expressed in trichoblasts and atrichoblasts.

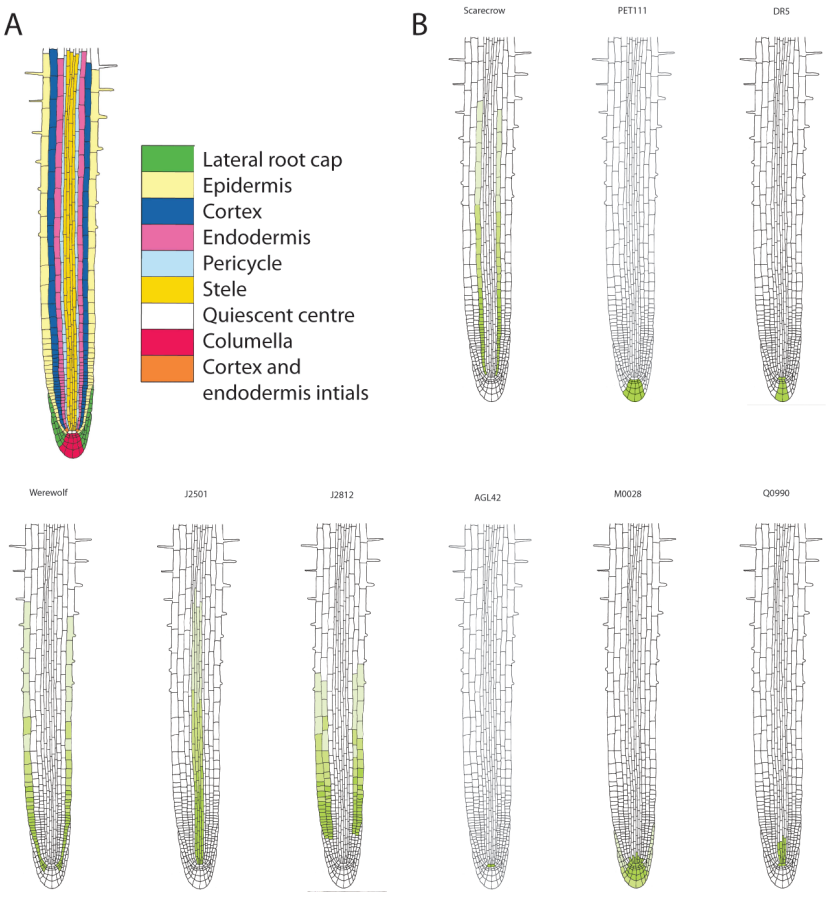

Figure 3. (a) The simple structure of the Arabidopsis thaliana root (b) Schematic overview of the cell type specific GFP expression patterns of some of the lines used for paper I and III.

3.3 Fluorescence Activated Cell Sorting (FACS) - Flow Cytometry Combined with Cell Sorting

FACS is a method designed to individually analyse and sort single cells based on their fluorescence, size and granularity. The FACS instruments used to produce the data in papers I, II and III are the FACSVantage® (Becton Dickinson), for early experiments, and FACSAria® (Becton Dickinson) for later experiments. The fundamental design for the two instruments is similar.

3.3.1 Technical Aspects of the Flow Cytometer

The flow cytometer can be divided into fluidics, optics and signal detection. In the fluidics system, cells or in the case of plants, the wall-less remains of digested cells called protoplasts, undergo hydrodynamic focussing. In hydrodynamic focussing, the sample, which is dispersed in a buffered solution, usually a phosphate buffer, but in our case a 0.7% saline solution, is introduced into the flow cell in the middle of a pressurized stream of sheath fluid. In this process, the cells are separated, aligned and carried to the point where they are intercepted by the lasers.

The FACSAria® instrument is equipped with three lasers, a 405 nm (violet), a 488 nm (blue), and 633 nm (red). When the cells, stained or unstained, pass the lasers the fluorochromes absorb energy and are excited. When the fluorochromes return to their ground state some of the energy is released as light, known as fluorescence. The light that is scattered axial to the laser beam is called forward scatter and indicates the relative difference in the size of the cells. Light scattered perpendicular to the laser is called the side scatter and it indicates the relative inner complexity or granularity of the cell.

The emitted fluorescent light is collected and focused individually for the individual lasers. It passes through to photomultipliers (PMT) that are equipped with filters that modify the spectral distribution of the light that reaches the detectors. Neutral density filters in front of the forward (FSC) and side scatter (SSC) detectors reduce the intensity of all transmitted light equally. A fixed percentage of the light is then passed on to the detectors. The long pass filters let wavelengths longer than the long pass filters specification pass through. Shorter wavelengths are reflected on to the next PMT that is equipped with a different filter. By contrast, band pass filters transmit only a set narrow range of wavelengths. Longer and shorter wavelengths are reflected.

In order to produce identical sized droplets of the sheath fluid-sample mix, a drop drive energy is applied to the stream of sheath fluid. If a cell passes the lasers and meets the defined sorting criteria set by the user it can be sorted into a positive sample collection tube. In order to do this, an electrical charge is applied to the stream just as the cell-containing drop breaks off the flow stream. When the drop brakes off the stream it retains the charge while flowing in the air. The drop will pass by two electrically charged plates and electrostatic repulsions and attractions generated by these plates will direct the drop towards collection tubes. The uncharged drops will not be affected and continue down in the centre and end up in the

waste (Robinson & Grégori, 2007). The protoplast populations destined for sorting in the studies presented here were gated according to figure 4 A-I.

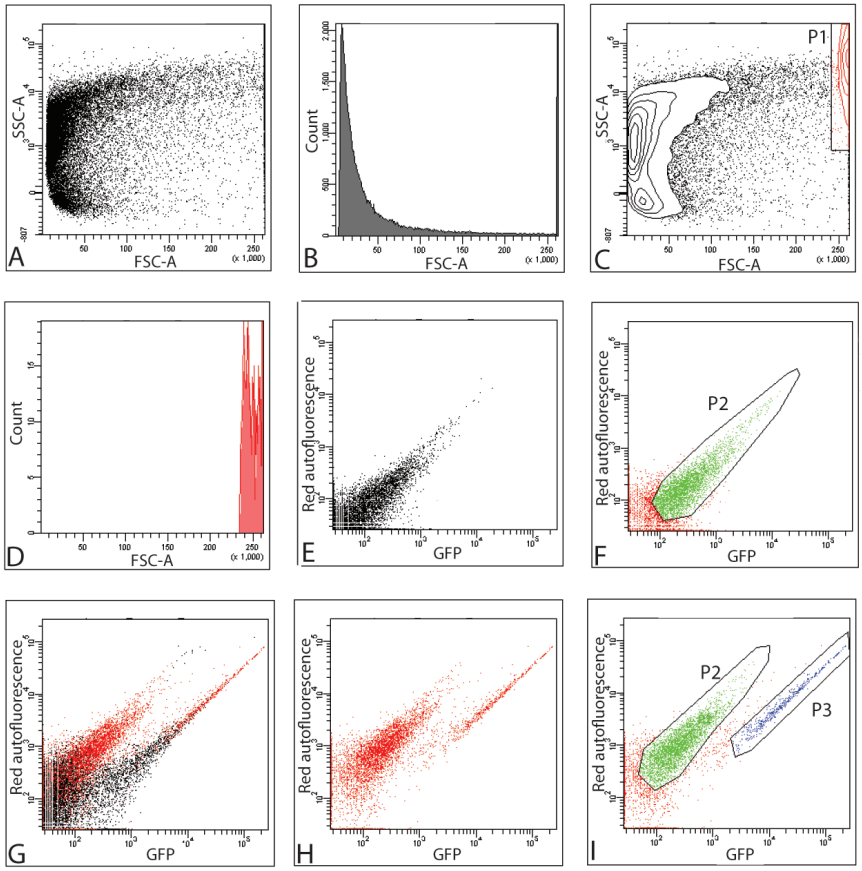

Figure 4. Gating for protoplast sorting. First, locating the protoplasts (A, B), The majority of events have a very low FSC, indicating a lot of small debris. The protoplasts, the P1 population, can be seen as a narrow line along the right Y-axis in (B) and more clearly when P1 (A) is presented as a contour plot (C). Only P1 events are shown in (D).

Second, defining a negative population. In (E) red auto fluorescence (PE-Texas red channel) of protoplasts with no GFP expression is plotted against GFP expression (FITS channel) and used as a GFP negative reference. P1 alone is shown in (F) and within P1, P2 is gated as the negative population.

Third, gating the GFP and the negative populations in a real sample. All events are shown in (G) GFP plotted against Red auto fluorescence P1, the protoplasts, shown in red. All events but P1 are removed (H) and the negative, P2 (green), and positive, P3(blue), events are gated for sorting (I).

3.3.2 Challenges using FACS for Sorting Protoplasts

Mammalian cells, which flow cytometers were originally designed for, are different from plant cells in three important aspects. First they are much smaller, second they are not as fragile as protoplasts, and third they can be more easily separated from each other without rupturing. Whereas a number of plant research groups have used FACS in their work, the most common applications for FACS in plant science are still cell cycle analyses, ploidity analysis and chromosome analysis (Pfosser et al., 2007; Barow & Jovtchev, 2007, Dolezal et al., 2007). Mammalian cells are typically 10-20 um in diameter. Most plant cells range from 20 to 100 um, Arabidopsis root protoplasts range from about 10 to 60 μm in size.

The FACS nozzle orifice should be at least twice the size of the largest particle to be sorted. The most commonly used nozzle for FACS is the 70um nozzle. Sorting with this nozzle enables rapid, up to 40000 sorting events/sec, sorting. Once the stream is stable, a drop delay can be carried out: i.e. determining how long a time it takes for the cell to go from laser interception and analysis to the point where it has been charged for sorting. Plant protoplasts are normally too large for the 70 um nozzle, and the 100 um nozzle is used instead. In order to be successful, sorting has to be done at a slower rate with the 100 um nozzle and with a lower pressure. One difficulty is that under these conditions, satellite drops form more easily and the stream is more unstable. Having satellite drops in close proximity to the drop of interest will increase the risk of sorting the wrong droplet. However, the proprietary FACSFlow™ buffer (composition partially unknown) appears to make the run smoother and the stream more stable.

3.3.3 System Stability is Dependent on a Clean Protoplasts Suspension

Plant protoplast preparations always contain a lot of debris originating from the isolation process i.e. cell wall debris (figure 4A,B). The debris can aggregate and clog the instrument, which ultimately requires restarting and recalibrating the settings. This can take from 20 min up to several hours, depending on the extent of the clog. If the clog can be fixed rapidly, within 15-20 minutes, the sorting can be continued, otherwise if the sample has been left too long on ice it needs to discarded. Mechanically trying to release protoplasts from the tissue as suggested by Birnbaum et al. (2005) increases the amount of debris significantly and is not recommended, particularly for cell samples with small populations of fluorescent cells i.e. columella or QC cells.

The degree to which the sample contains debris affects the sorting rate considerably. A lot of debris results in a high event rate, but not all events are cells, leading to a low sorting rate. Increasing the sorting rate reduces the sorting efficiency because of too many conflicting events. If a protoplast batch with a high event rate and few GFP positive protoplasts is to be sorted, it will either be accurate high yielding and take a long time, or be faster with a lower yield. A low yield means that many positive protoplasts will be lost, as they will not be sorted. One constant is that the time for isolation and sorting should be minimised as far as practicable, as delay affects sample quality negatively.

When a protoplast containing droplet is charged and destined for sorting it ends up in the collection tube. The first protoplasts will collide with the tube wall and burst. This occurs until the bottom of the tube is covered by liquid. Adding 500 µl buffer to the collection tube prior to starting the run solves this problem. Broken cells were not a problem for IAA quantification, however, as the IAA from both intact and broken cells was extracted on the SPE columns. Re-sorting of collected protoplasts showed that approximately 75-80% remain intact after the first sorting. The contents of the 20-25% of protoplasts that break during sorting can be removed by spinning down the protoplasts after sorting, with subsequent removal of the buffer. Centrifugation and removal of the buffer will also damage some protoplasts and care should therefore be taken during this process. If the extraction process cannot be made directly in the collection tube, another point for potential loss of material is introduced in decanting the protoplasts. For transcriptomics, the effects of these problems can be somewhat overcome by amplifying the material before the array analysis.

3.3.4 Obtaining Enough Sorted Material

The sum total of all of the points for loss or damage of material means that the actual yield is only a fraction of the theoretical yield. Collecting enough material quickly, particularly for protoplast types that compose only a small fraction of the total, is the major obstacle. The first point of loss occurs because all GFP positive cells are generally not released from the original plant material. During the sorting process, about 80-90% of all events are directly gated as debris and broken cells. There is also loss due to poor GFP expression in the cells. Many of the lines used in these experiments, such as the *GFP-WEREWOLF* line (Ryu et al., 2005) have weak GFP expression. This means that the GFP positive population and the reference population will not be sufficiently separated for efficient sorting. The lines used in the experiments were not originally constructed for FACS, but to be visible

under Confocal Laser Scanning Microscopy (CLSM). Furthermore, the expression pattern in many lines can be inconsistent, with the fluorescent signal overlapping different cell types in many instances. All of these potential drawbacks need to be taken into consideration and strategies for avoiding or minimising them need to be devised if the resulting data is to have real meaning. A considerable part of the work conducted for this thesis comprised finding solutions to these technical limitations.

3.3.5 FACS Flow™ as a Sheath Buffer Poses Problems

The FACS Flow™ is recommended for sorting by the manufacturer, BD. The complete composition of this buffer cannot be deduced from the material data safety sheet but it is said to be a PBS type buffer (pers. comm. Mike Cook, Duke Cancer institute, Durham NC). Metabolomics analysis has confirmed the phosphate content, however, it clearly contains other important, but undefined, compounds that appear to facilitate sorting through smoother and more stable fluidics.
For IAA analysis, the FACS Flow™ did not pose any problems, but for oxIAA analysis this buffer reduced the recovery significantly. Instead, we found that a 0.7% NaCl solution could be used successfully as a sheath fluid, particularly because it gave a higher recovery of oxIAA.

The FACS instrument works optimally with the FACS Flow™, and switching buffer makes the procedure more unstable and laborious. It also makes it more difficult to perform the cytometer set up and tracking (CST) and this increases the time of and variation within the sorting process. It is not possible to sort in water, which would otherwise be the best option with regards to the following analysis, as without some osmotic adjustment water would be drawn into the cells and the protoplasts would burst. There is also an absolute requirement for an ion in the buffer solution, in order to enable the charging of the droplets for subsequent sorting.

One of the main disadvantages of using a 0.7% NaCl solution is that the flow cell becomes damaged. Dirt and salts builds up on the inside that are difficult to remove with both standard and extended cleaning routines. The use of Deacon (Novakemi AB, Enskede), a strong solution of potassium hydroxide, to remove salts and dirt coating the inside of the flow cell has been suggested as a solution. It is not recommended for extended use as it etches the plastic of the flow cell, thereby reducing its lifetime. It is, however, efficient at cleaning the build-up of debris and salts. As a result of these problems, the flow cell has had to be replaced three times over the last four years. The challenges of finding a suitable buffer and solving problems

caused by non-optimal buffers have meant considerable added costs and just as importantly have been time consuming.

These considerations are relevant for all GFP lines sorted for paper I, II and III. Whereas the drawbacks listed here indicate the potential pitfalls in FACS sorting of plant protoplasts, in the hands of an experienced operator, if care is taken to minimise their effects the technique provides a powerful means for obtaining cell type specific information in plants.

3.4 Normalization Procedures and Considerations for Analysing IAA in Protoplasts

The most frequently used method of presenting metabolite concentrations is mole/g fresh weight or, in our case, per number of cells. This was not considered a suitable way to present the data in Paper I. Instead, the IAA levels are presented, as a ratio between the sorted GFP cell population and its reference population, i.e. all other cells sorted but not expressing GFP. We have used this method because the IAA content of the reference cell population is thought to be relatively stable compared to the GFP expressing cells. If the samples are prepared identically it allows a comparison of the concentrations in samples taken on separate occasions. It is vital to keep the sampling and sample preparation procedure identical as it took an average of 4-5 months to collect enough material for only one full set of samples covering the different cell types in the root apex analysed for Paper I. In total three full sets were collected.

Protoplasts isolated from cells in the root apex differed in size (10-60 μm in diameter), depending on the cell type from which they were isolated. The size of an intact cell does not necessarily correspond to that of its protoplast. Depending on cell type, the protoplasts may distend differentially when released from their cell wall into the protoplast isolation buffer.

As a result, comparing IAA levels in a fixed number of protoplasts could in theory mean that the weight of the samples differed by up to a factor of six. It is believed that IAA exists primarily in the cytosol, with some also likely to be in, or connected with the ER. It is unknown if IAA exists in other cellular compartments, however, the vacuole is potentially an important storage compartment for IAA and IAA conjugates (Krecek et al., 2009; Friml and Jones, 2010). Assuming an equal IAA distribution in the cell and its compartments, it was decided to normalise against the mass or weight of the collected protoplasts rather than number. It was thought possible that the size of an intact cell might not necessarily correspond to that of its released protoplast. That is, it was thought that it might be

possible that depending on original cell type, the protoplasts might distend differentially when released into the protoplast isolation buffer. Confocal Laser Scanning Microscopy (CLSM) was conducted on the GAL4-GFP lines and five representative lines were chosen for measuring intact cell volume. The calculated *in planta* cell volumes were compared to corresponding populations of protoplasts and it was concluded that protoplasts swell up proportionally to the original cell size. As a result, it was possible to use protoplast size for normalisation. Average protoplast volumes were calculated to mg, i.e. how many protoplasts, if we assumed a density equivalent to water, would equal 1 mg tissue? Theoretically each GFP line would have its own average size reference population, as the GFP population in question should be subtracted from the total average. For most lines this did not make a significant difference. Consequently, all GFP lines have the same reference population size. If IAA content were relative to protoplast size i.e. higher IAA content in a larger cell, this recalculation would compensate for that factor.

All samples were analysed in pairs, i.e. GFP and a reference population, which constituted all other cells in the root. In practice, this meant that the control population consisted of all other cells that were released from the root during protoplast isolation and were sorted as GFP negative. The absolute IAA content varied considerably between replicates but the relative ratios between the GFP sample and the reference population was reasonably stable, supporting the use of this normalisation procedure. The IAA levels in Paper I are depicted as a ratio between the GFP population IAA content and the reference population IAA content. Apart from different phenotypes, C24 and Colombia also have different compositions of IAA and its precursors (Petersson, 2010 unpublished data). Therefore, normalisation was conducted to reduce differences originating from the use of two different ecotypes (Columbia and C24). In order to see if the normalisation carried out for protoplast size was reasonable, the average number of cells in the apical 1 mm of a root model was calculated based on CLSM cell size data. It was then multiplied by the average protoplast IAA content and compared to the apical 1 mm of sectioned roots. The calculated IAA concentration was only twice as high in the protoplast based root model as in the apical root section, concluding that the normalisation was realistic.

3.5 Instrumentation for Mass Spectrometry

Mass spectrometry is a widely used method for the detection, identification and quantification of compounds such as metabolites, proteins and peptides.

The initial step, sample separation, is carried out by either gas (GC) or liquid chromatography (LC). GC is the method of choice for smaller ions that can be vaporized without decomposition, whereas LC is generally better for large biological molecules. Ionization is a critical step in mass spectrometry as only ions can be can be accurately measured. Once an ion's mass is known, the chemical composition can be deciphered based on its fragmentation pattern.

3.5.1 Gas Chromatography Coupled to Magnetic Sector MS

Most IAA samples were analysed by GC-SRM-MS using a JEOL-MStation instrument. In GC, compounds are separated based on their boiling point and interactions with the stationary phase of the column and only volatile compounds can be separated by GC. Compounds that are non-volatile must first be derivatized. For IAA analysis, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and methylation are used for derivatisation. BSTFA makes the analyte less polar, more volatile and thermally stable.

In GC, samples are introduced into the GC inlet, which is set at 250°C. This evaporates the sample before it enters the GC column. The length and polarity of the GC column can be varied depending on the application. For IAA analysis a non-polar DB5 (20 m) column was used.

The evaporated analyte exits the GC column and enters the ion source where it is ionized by electron ionisation (EI). EI is good for relatively small volatile compounds. Electrons are produced by heating a rhenium filament in the ion source. Once produced, they are further energized by acceleration through a potential of 70V. The electrons are focused to a beam by a magnetic field. The electron beam interacts with the column eluent and positively ionises the molecules as they emerge. This causes the molecules to fragment.

The resultant positively charged ions next enter the sector analyser and the first electric sector where they are focused but no separation occurs. The focused ion beam then enters the magnetic sector where the ions are deflected according to their mass to charge (m/z) values. Ions with individual m/z values can be selected by changing the strength of the magnetic field. The selected ions are then focused again in a second electric sector and detected by an electron multiplier. IAA analysis in paper I and III were performed as described by Edlund et al. (1995).

3.5.2 Liquid Chromatography Coupled to Triple Quadrupole Analyser

For paper II The samples were analysed by an Agilent UPLC-ESI Q^3MS. LC, the sample constituents are separated on a column based on their polarity. C18 columns were used in the work presented in this thesis. The retention time depends on the strength of the analyte's interaction with the non-polar stationary phase. Running a mobile phase gradient, i.e. gradually increasing the amount of organic solvent to water, reduces the polarity during the course of a run.

The LC eluent is next introduced into the ion source. Electrospray ionization (ESI) is used for ionization. ESI is a superior ionization technique, especially for large non-volatile biological molecules such as proteins and peptides, as it is a soft ionization process. It is called soft because the ionized molecule does not fall apart or break-up during the process. Electrospray ionization (ESI) generates a liquid aerosol through electrostatic charging. The liquid is passed along a capillary tube to which a strong electric potential is applied. When the liquid reaches the end of the capillary tube the electric field causes it to be nebulized into a cloud of very small droplets. The droplets rapidly shrink in size as the solvent evaporates. The distance between electrical charges decreases until the electrical charge reaches a critical state and the natural repulsion between charges causing the release of ions and neutral molecules (Herbert & Johnstone, 2003). The ions are then introduced into the triple quadrupole mass analyser. In a quadrupole mass analyser, electric fields are used to separate the ions based on their mass. The quadrupole consists of four circular parallel rods that have fixed direct current and alternating radio frequency (RF) potentials applied to them. Ions produced in the source of the instrument are then focussed and passed along the middle of the quadrupoles. The motion of the ions will depend on the electric fields, so that only ions of a particular m/z will have a stable trajectory and thus pass through to the detector. The RF is varied to bring ions of different m/z into focus on the detector. The first and third quadrupole function as mass filters and the second as a collision cell (Hoffman & Stroobant, 2002).

3.5.3 Gas Chromatography–Time Of Flight-MS

An Agilent 6890 GC coupled to a Pegasus III TOF-MS instrument was used for the metabolomics analysis. Samples for metabolomics analysis are separated by GC and ionised by EI as described above and analysed using a Time Of Flight (TOF) analyser, which is based on the time it takes for a singly charged ion to travel the length of the flight tube, which is

proportional to its mass. Accelerating a pulse of ions in a flight tube will give the ions a velocity that is proportional to their individual m/z values. The detector records the arrival times and the flight times can be converted to masses (Herbert & Johnstone 2003).

3.5.4 LC-QTOF-MS for Proteomics

The proteomics samples were analyzed by reverse-phase LC-ESI-MS, using a nanoACQUITY ultra-performance liquid chromatography (UPLC) system coupled to a Q-TOF mass spectrometer, Q-TOF Ultima; Waters Corp. A Quarupole Time Of Flight (QTOF) instrument is a hybrid of two mass spectrometers where the quarupole is used to select the ions to be examined and the mass spectra is generated by the TOF.

3.6 Summary Methods

I have developed a method for cell specific IAA analysis, where I combine the use of Arabidopsis lines with cell type specific GFP expression, Fluorescent Activated Cell Sorting and high resolution Mass Spectrometry. Individually, the use of these techniques are widely accepted but combining them for analysis of the plant hormone auxin on a cellular level has not been previously conducted. We now have a robust method to perform this type of analysis and we believe that the uncertainties of the generated data are associated with biological variation and not the method itself. Being able to follow auxin distribution on a cellular level has many interesting potential applications. For example, for determining how auxin distribution changes during development, under pathogen attack, in auxin mutants and over time in inducible systems. As it is also possible to sort plant cell organelles by FACS, this methods also provides a powerful tool to further understanding the subcellular localisation of IAA. This method can of course also be used analysis for other compounds that are present in the protoplasts in a sufficiently high concentration such as other hormones and signalling molecules.

4 Measuring and Regulating the Levels of Free IAA

As described in chapter 1, IAA distribution and concentrations depend on synthesis, degradation, conjugation and transport. Environmental stimuli and interactions with other hormones can initiate and regulate these changes. In this part I will first present an IAA distribution map with cellular resolution (paper I). I will then discuss how catabolism, by converting IAA into its major catabolite oxIAA, can contribute to the regulation of the levels of free IAA (Paper II), and finally I will describe how IAA and CK homeostasis are interdependent and affect IAA biosynthesis and levels (paper III).

4.1 Paper I – Pushing the Limit of Detection and Confirming the IAA Gradient

In paper I (Petersson et al., 2009) we push the limits of detection, and show the distribution of IAA in the root at cellular resolution for the first time (figure 5A) We have now reached a point where we can quantify IAA levels on a tissue and cell specific level. In 1990, Sandberg et al. used 1 kg of *Nicotiana tabacum* leaves to quantify IAA. In 1991, Sitbon et al. used 1 g of *Nicotiana tabacum* leaves, and in 1995 Edlund et al. used only 1-10 mg *Nicotiana tabacum* leaf discs to quantify IAA. Now we do routine analysis on samples containing only 200,000 protoplasts. Although it is possible to quantify IAA in as few as 10,000 cells, we avoid using such small sample sizes in exchange for superior chromatography and peak shape. One advantage with IAA extraction from protoplasts is that the extractable portion of IAA is more accessible compared to intact tissue, where incomplete homogenisation of the tissue could reduce the amount of

extractable IAA. Obtaining complete homogenisation is especially difficult in small samples, as the volume of extraction buffer (500 µl buffer in a 1,5 ml tube and a 2 mm tungsten bead) compared to the sample size (e.g. 10x5mm root apices as in paper II) is very large.

Local auxin distribution patters at a resolution comparable to that achieved in paper I has previously only been estimated by mathematical and computer modelling or by reporter genes. Here we show analytical data confirming an IAA gradient in the rot apex (figure 5A).

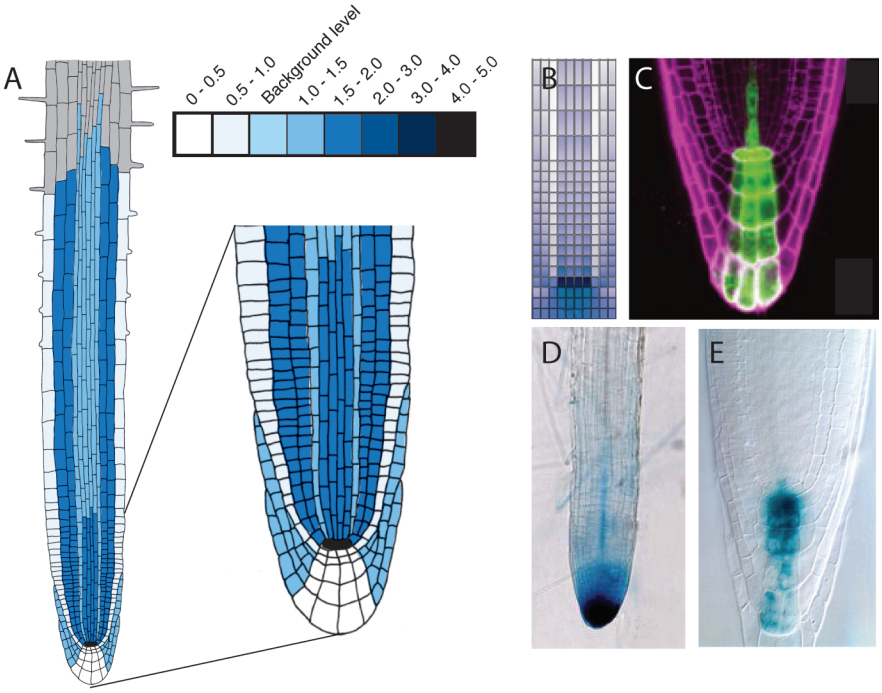

Figure 5. (A) IAA distribution pattern in the root apex, based on analytical data from sorted protoplasts and MS analysis. (B) IAA distribution in a schematic root based on mathematical modelling. (C) IAA "distribution" visualised by the DR5-GFP reporter gene construct. (D,E) IAA "distribution" visualised by the DR5-GUS reporter gene construct.

There are two examples where IAA has been quantified in thin sections of the vascular cambium in pine and hybrid aspen where they describe an IAA gradient over the cambium at very high resolution. However, these authors do not reach cellular resolution as each section is more than one cell thick (Uggla et al., 1996; Tuominen et al., 1997).

An IAA transport model, containing spatial patterns of auxin concentrations in the shoot apical meristem (SAM), was published by

Jönsson et al. (2006). Based merely on data for the localization and properties of the efflux carrier PIN1 it was possible to create this pattern. It was followed by a study where the importance of the influx carried AUX1 for maintaining high IAA levels in meristem SAM epidermis was explored by mathematical modeling (Heisler & Jönsson, 2006). They found that AUX1 plays a crucial role in maintaining high auxin levels in the SAM epidermis. AUX1 has been shown experimentally to play a vital role in the supply of IAA to developing root hairs (Jones et al., 2009). When IAA distribution in the root was modelled, it was found that the root was able to generate an auxin maximum around the quiescent center and a highly robust gradient. The model was based on internal PIN distribution and basic properties such as diffusion and permeability. The modeled maximum could be formed without IAA synthesis, degradation or regulated influx (Grieneisen et al., 2007) (Figure 5B). According to data from paper I, that shows that the IAA synthesis levels are approximately the same in all cell types in the RAM, we could confirm the Grienesien model on the point that differences in cell type specific synthesis are not necessary for establishing the IAA distribution pattern seen in the apex. The amount and orientation of IAA transporters (PINs, AUX1), that these models are based on, do not however reveal anything about the actual IAA concentration, only the direction of the flow of free IAA.

A number of attempts have been made in the past to report auxin concentrations indirectly. Various researchers have used marker genes such as GFP or GUS driven by artificial or native promoter sequences that are responsive to auxin as proxy reporters of auxin levels. The DR5-GFP reporter is an artificial auxin responsive promoter element (AuxRE) driving the expression of GFP. Together, these elements provide an indication of auxin responsive transcription in cells and tissues (Ulmasov et al., 1997). Various versions of the DR5 artificial promoter exist, however, the DR5-GFP reporter is usually strongly expressed in the QC and the initial columella cells (Wolverton et al., 2011) (Figure 5c). It has from time to time incorrectly been used as an IAA concentration reporter, for example by Jones et al. (2009). Depending on weather DR5 is coupled to GFP or GUS the patterns are different and the different versions of the promoter are active in different tissues and at varying levels. The DR5-GUS staining patterns are different depending how long you stain. The staining patterns range from only being detected in the QC and the upper columella cells, to the whole meristematic region and to also including vascular tissues (Figure 5 D,E).

Auxin distribution in the plant root system and rosettes has been visualised by IAA2-GUS localisation (Rusak et al., 2010). The SAUR-GUS promoter reporter responds to IAA in a dose dependent fashion and has been used to pinpoint the localization and degree of auxin response (Li et al., 1991). What all of these reporters have in common is that they report levels of responsiveness of particular promoter elements in individual cell types, not on the levels of the hormone itself. As has been shown here, the level of responsiveness in a particular cell type is dependent on a broad range of factors, including the capacity of the individual cells to respond, depending on the presence or absence of auxin response elements.

As established by Ljung et al. (2001), young developing tissues like meristems and young leaves have the highest capacity to synthesise IAA. The QC and the initials, the origin of all cells in the root, are shown to have the highest IAA levels in the root apex. The physical distribution of IAA in the root apex shown in paper I (figure 5A) concurs with the notion that the QC has the highest IAA levels as shown by modelling by Grienesien et al., 2007 and speculated by indirect evidence from DR5 data (Ulmasov et al., 1997).

The point of greatest contention is where the modelled and DR5 indicated IAA distribution diverge from what is presented in paper I, in the columella region. As seen in paper I, there is a lower than reference, but not in any way absent, level of IAA in the columella compared to the reference cells. The columella has previously been thought to contain high levels of IAA based on modelling and DR5-GUS reporter gene expression. This may be explained by the transport of IAA down the root in the vascular tissue towards the QC bottleneck, where all IAA passes through to the columella. The IAA flow is quickly redirected or perhaps degraded in the columella towards the epidermis and cortex, thus high IAA concentrations do not have the time to build up in the columella. All these processes involve active transport, which the Grienesien model is based on. The DR5 reporter gene is base on the activation of an auxin response element. Many IAA molecules pass through the tissue and have the possibility to activate these response elements. Although IAA is also synthesised in the region it does not necessarily mean that the concentration has to be high. Currently, paper I is the only paper describing the IAA distribution with high resolution in the root based on actual measurements.

Paper I confirms the existence of an IAA gradient within the root apex. The IAA levels increase in the vascular tissue towards the QC (figure 5A). The QC has the highest levels of IAA and cells adjacent to the QC, the stem cells, have lower but still high levels of IAA and the IAA level

decreases the further away from the QC you get. IAA gradients are important for the initiation of the formation of new organs. It determines the developmental fate of cells and target genes in a concentration dependent manner (Bhalerao & Bennett, 2003; Benková et al., 2009). For example, a high resolution IAA gradient over the cambial zone was observed in both pine (Uggla et al., 1996) and hybrid aspen (Tuominen et al., 1997) peaking in the meristem, where the cells are actively dividing. It has also been shown that the expression of auxin-responsive genes responds dynamically to changes in the cellular auxin concentration over the IAA gradient seen in hybrid aspen (Nilsson et al., 2008).

After many years of analysing the IAA content in Arabidopsis root protoplasts, it is clear that the absolute concentrations of IAA in biological replicates can vary considerably, but that relative ratios between cell types remains relatively stable. There are many possible sources for the variation in IAA content such as biological differences derived from different ecotypes, changes in growth conditions in the climate chamber, the isolation and sorting procedures, how well the plants were doing at the time of harvest, the possibilities of undetected microbial infections, and so on.

The data presented in figure 5A supports the idea that it is the gradient itself that provides the developmental cue to the cells and tissues, and not the absolute IAA concentrations.

What is then the optimal resolution to present IAA distribution in relation to the available technology and methodological limitations? With the development of more and more sophisticated analytical instruments, with increasing resolution and sensitivity, we have gone from looking at whole plants to single cells.

Taking a whole plant and extracting metabolites from a mixture of roots, stem, leaves, flowers and siliques may be as informative and imprecise as grinding up and extracting a whole human in order to see if he or she has diabetes. With this approach it is only possible to detect and quantify the metabolite in question, in this case IAA, and at most to do a time series to track hormone dependent developmental changes over time and total hormone levels over time. But it does not say anything about how and where IAA is localised, how the absolute hormone levels reflect developmental changes, or even if they even do. When analysing whole seedlings of IAA mutants for IAA levels, important IAA differences could be masked by a dilution effect from other tissues. In Barlier et al. (2000), whole plants of the IAA over-producing mutant s*uperroot 2* (*sur2*) were analysed, showing a clear increase in the overall IAA content. It is, however, not known if this is a general increase, or if there are distinct tissues where the

IAA concentration is particularly high or low. Taking one organ at a time i.e. leaf, root or stem, will add a dimension to the picture. It will be possible to observe if organs, with different purposes, have different IAA requirements. It has been suggested that IAA gradients in a tissue play a role in regulating developmental responses (Uggla et al., 1996, Tuominen et al., 1997). The Arabidopsis root and leaf have been subdivided into sections confirming that IAA gradients exist (Ljung et al., 2001, 2005). Now, as a result of the work presented here we have a way to examine one cell type at a time and this has provided us with a map of the physical distribution of IAA within the root apex. By extending and further refining this work, in the future we will be able to ask questions regarding IAA related development in the root on a cellular level.

4.2 Paper II – Regulation of Free IAA Levels by Degradation to oxIAA.

The aim of this work was to test the hypothesis that non-decarboxylative oxidation of IAA to oxIAA is essential for the establishment and maintenance of the IAA gradients and maxima regulation in the root apex. Most research on the catabolism of IAA has focused on IAA conjugation, rather than on oxidative degradation (Woodward & Bartel, 2005). Several publications, however, show that oxIAA is a major IAA catabolite and that by contrast IAA conjugates are present only in very low concentrations (Kowalczyk & Sandberg, 2001; Östin et al., 1998; Kai et al., 2007). The IAA gradient in the root is generally thought to be formed and maintained by active transport of IAA from the aerial part of the plant and by recirculation of IAA in the root apex (Grieneisen et al., 2007). With a continuous supply of IAA to the apex the IAA concentration would increase, but it does not. There must be a mechanism to maintain the IAA concentrations on an appropriate level. We suggest that conversion of IAA to the inactive catabolite oxIAA reduce the IAA levels and help to maintain the IAA gradient.

The work described in Paper II reinforces the previous findings, showing that oxIAA is the major IAA catabolite in Arabidopsis. Quantification of the levels of IAA, oxIAA and the two major conjugates IAAsp and IAGlu in the root tip showed that IAA and oxIAA levels were similar, but the level of IAAsp and IAGlu were an order of magnitude lower (Fig 3, paper II). When excised roots were fed with radiolabelled IAA in agar to the surface of the scission, it was basally transported and rapidly oxidised to oxIAA and oxIAGlc (Fig 4, paper II). Increasing the endogenously IAA levels by

feeding whole seedlings with IAA in liquid culture resulted in an accumulation of oxIAA and IAGlc in the root (Fig 5, paper II).

IAA conversion to oxIAA is irreversible, and the catabolite does not have IAA activity. Feeding *DR5rev:GFP* Arabidopsis lines with oxIAA fails to induce GFP expression in the short and long (24 hours) term (Fig 7, paper II). IAA inhibits root elongation in a concentration dependent manner. Feeding roots with oxIAA at concentrations that for IAA completely inhibited root elongation had no effect on root growth (Fig 8, paper II). An *in vitro* binding assay also indicated that oxIAA does not bind to the TIR1 auxin receptor (Fig 9, paper II). Together, these data strongly indicate that conversion of IAA to oxIAA is an irreversible mechanism for reducing the levels of free IAA in cells and tissues, and that oxIAA do not exert any IAA like effect.

It can be hypothesised that as all cell types analysed in the root apex are able to synthesise IAA (Petersson et al., 2009), they are also able to oxidize IAA as an integrated part of an effective regulatory mechanism. It is plausible that some cell types have a greater capacity than others to synthesise IAA under certain conditions and different developmental stages. Likewise is it also conceivable that certain cell types have an increased ability to oxidize IAA in order to fine-tune the distribution of IAA (paper I).

Whereas IAA is continuously synthesised in all cell types, in approximately the same amount and rate (papers I and III), IAA oxidation might be a more responsive mechanism (Fig 4, paper II).

IAA is synthesised in Arabidopsis via a number of independent but interconnected pathways, as described in the introduction. Several of the early steps in IAA biosynthesis are also branch points for the biosynthesis of related compounds such as indole glucosinolate and camalexin. Reducing IAA by oxidation could be used by plants as an alternative to reducing IAA synthesis rates. As many of the IAA precursors are essential for the biosynthesis of other compounds, this could have negative effects in the overall state of the plant.

We aim to quantify the cell type specific levels of oxIAA in order to establish whether the IAA distribution pattern seen in paper I is at least partially the result of cell specific IAA degradation, or whether it is derived solely from the active re-localisation of IAA through transport pathways.

A method has now been established whereby the concentrations of IAA and oxIAA can be quantified in isolated protoplasts. This method will allow for the determination of the ratio between these compounds in specific cell types.

The absolute IAA concentrations, as measured for paper I, vary but the relative levels between GFP positive populations and reference populations for specific lines are rather constant. Preliminary data show that this does not appear to be the case for oxIAA, suggesting the existence of a rapid, cell specific mechanism for degradation of IAA to oxIAA. Thus the cellular distribution of oxIAA seems to be highly dependent on the current status of the plant, which may vary slightly between replicates because of biological variation even under the best possible sampling conditions.

In intact roots, the oxIAA:IAA ratio is relatively constant, with an almost a 1:1 ratio (paper II fig 3). By contrast, preliminary data show that the oxIAA:IAA ratio is not 1:1 in isolated protoplasts from lines expressing GFP in different cell types. In biological replicates, of the same sorted cell type, the oxIAA:IAA ratio ranged from 0.5:1 to 6:1. The overall root oxIAA:IAA ratio may, however, still be 1:1 if one considers the average of all cell types. But on the level of a specific cell type is seems as the oxIAA:IAA ratio is very dynamic. This variation in the oxIAA:IAA ratio could be the result of cell type specific dynamic changes regulating IAA levels.

In order to determine if the conversion of IAA to oxIAA required *de novo* protein synthesis, plants were incubated for 6 hours in the protein synthesis inhibitor, cycloheximide (CHX). The preliminary data showed that the treatment led to a slight reduction in IAA levels and an almost doubling of the oxIAA levels. Levels of the IAA precursors, IAN and tryptamine were clearly reduced by the CHX treatment. The data suggest that *de novo* protein biosynthesis is not required for oxIAA formation, however, the data also suggest the possibility that catabolism of oxIAA requires newly synthesised proteins.

Material for cell type specific measurements of oxIAA has sorted over the course of several years. Over this time, the analytical instruments have been replaced, extraction protocols have been further developed and different people have been involved in protoplast isolation and sorting, extraction and analysis. Initially, the oxIAA:IAA LC-MS analyses were made in collaboration with the Laboratory of Growth Regulators in Olomouce, Czech Republic. An in-house LCMS protocol has now been established for these analyses. A high-resolution UPLC-MS instrument dedicated to the analysis of low abundance molecules is also now available. One of the main changes in terms of protocol development has been that the original FACSFlow^{TM} sorting buffer has been replaced by a 0.7% NaCl buffer because it gave a better recovery. So far we have not been able to produce consistent cell type specific oxIAA:IAA ratio data (data not shown). It is

likely that the variation in the data is due primarily to the variations over time in the sample collection, preparation, extraction and analysis.

In addition to the variations in sampling extraction and analysis, the levels of the different IAA metabolites are likely to be affected by other experimental variables. For example, IAA is actively re-localised in roots as a result of changes in the gravitational vector (Muday, 2001). What affect the suspension of the roots in cell wall degrading isolation buffer for 1.5 hours has on auxin content in the various cell types is unknown. Other factors that might affect IAA levels include the excision of the aerial portion of the seedling, dispersion of the roots in liquid media, and enzymatic degradation of the cell walls. It will be a challenge to find the right conditions for growth, sectioning and protoplast isolation that will reduce the variation to a minimum. However, despite the many technical challenges it is expected that continued technical refinements will eventually make it possible to determine the distribution of oxIAA in the root apex at a cellular resolution.

Questions that will hopefully be answered in the future by further refining and applying the techniques presented here include: What happens to oxIAA and where is it formed and degraded? The IAA:oxIAA ratio is relatively stable when measured in intact tissues, but not when measured in protoplasts. One possible explanation for this is that the apoplast houses an important feature in regulating intracellular IAA concentrations?

4.3 Paper III – Cytokinin Regulation of Auxin Biosynthesis

Paper III (Jones et al., 2010) describes research aimed at understanding the influence that CK has on IAA biosynthesis. Previous research (Eklöf et al., 1997; Nordstöm et al., 2004) had shown that IAA directly influences CK biosynthesis and that CK in turn influences IAA biosynthesis. Together, these data suggest a feedback biosynthetic regulatory loop between CK – IAA biosynthesis.

Increasing the CK content by inducing the expression of CK biosynthesis gene *IPT8/pga22* in a transgenic inducible line increased the biosynthesis rate of IAA was performed in paper III. It appeared that it was primarily the young developing tissues such as the shoot and root apices that had this ability to respond to changes in hormone content, and not the mature tissues such as mature leaves. This data on young tissues contradicts previous data from this lab Eklöf et al. (1997) and Nordstöm et al. (2004) where the opposite relationship was found, i.e. increasing CK led to reduced levels of IAA. However, these measurements were taken from older plants and where the whole plant was sampled. The feeding times were also

substantially longer and cannot be directly compared. The reason why no differences were observed when whole plants were used may be explained by the fact that the young actively dividing tissue would only comprise a small fraction of the whole sample and the differences would be indiscernible because of a dilution effect.

One possible explanation for the mature tissues not showing the response is that they may be more resilient when faced with sudden changes in hormone levels than developing tissues, as they have already passed critical stages in its development where it is imperative that the hormone levels are precisely tuned for the developmental process that are occurring in young tissues. Adding CK exogenously also resulted in an increase in the biosynthesis rate of IAA in young developing tissues. The specific work that I conducted within this project was to determine if the effect of increased CK is a general increase in the IAA biosynthesis in all young actively growing tissues or whether there are cell type specific differences in the response. Measurements of IAA biosynthesis rates induced by cytokinin revealed that all of the different cell types had similar IAA synthesis rates and therefore did not appear to be differentially affected.

Interestingly, decreasing the CK levels by inducing the expression of an inducible transgenic *CYTOKININ OXIDASE* (*CKX*) reduced the level of IAA biosynthesis in young developing tissues. The same result was observed in Adenosine phosphate-isopentenyltransferase (IPT) mutants that are defective in CK biosynthesis. Together, these data indicate that CK is a key regulator of IAA biosynthesis.

Feeding studies (paper III) with auxin and cytokinin signal transduction mutants indicated that both the IAA and CK signal transduction mechanisms are involved in the CK regulation of IAA biosynthesis. Based on IAA biosynthesis measurements in various mutant and transgenic plants and transcriptional profiling, a model was proposed where CK is a positive regulator of IAA biosynthesis and IAA is a repressor of CK biosynthesis. Transcriptional profiling data in paper III indicate that IAA and CK regulate each other both through signalling, transport and degradation.

IAA and CK concentrations and biosynthesis levels can be regulated in different ways. As described in the introduction, constitutive over-expression of IAA and CK related genes were carried out by Eklöf et al. (1997). Feeding plants with exogenous IAA and CK was carried out by Nordström et al., (2004) and, in the work described in paper III, a CK inducible system was used. Whereas it is difficult to compare the data from one experiment to another directly when there are significant differences in how the hormone levels are increased, the timing of treatments, the age of

the plants, the type of sampled tissue and how it is collected, all three papers point in the same direction, that there is interdependency in terms of hormone biosynthesis between IAA and CK. The importance of these findings is suggested by the large amount of data that has shown the critical roles played by interactions between auxin and cytokinin in a number of developmental processes in plants. The interaction between IAA and CK has been shown to be crucial for the plant to specify the root stem-cell niche (Müller & Sheen, 2008). For example, the transcription of ARR7 and ARR15 (negative regulators of the cytokinin signalling) are induced by an auxin maxima in the root tip during early embryogenesis. This leads to suppression in the CK output in the early embryo, which is necessary for the plant to specify the root stem cell niche (Müller & Sheen, 2008). The opposite relation for IAA and CK has been found in the shoot apical meristem, where auxin represses the ARR7 and ARR15 and CK induces their expression (Zhao et al., 2010). Not only is the specification of the root apical meristem (RAM) dependent on IAA – CK crosstalk, but also the differentiation of the root meristem is tightly regulated by the IAA – CK interactions. CK induces cell differentiation in the RAM, thereby inhibiting further cell division, and thus RAM size in negatively regulated (Dello Ioio et al., 2008). Adding CK to a wild type plant will result in a smaller RAM and CK biosynthesis and signalling mutants have larger RAMs (Dello Ioio et al., 2008). This connection was established when ARR1 was shown to activate the SHY2/IAA3 protein, an auxin signalling repressor, that inhibits the expression of the auxin efflux carriers, PIN 1, 3 and 7. The reduction of PIN 1, 3 and 7 content makes the cell unable to respond to auxin-induced cell division in the RAM (Dello Ioio et al., 2008).

4.4 Summary and Conclusions

We were able to confirm the existence of IAA gradients in the root apex. Whether it is the actual IAA gradient that dictates the developmental fate of cells is yet to be discerned. The IAA gradient is most likely maintained not only by active IAA transport but also by IAA biosynthesis and catabolism. The conversion of IAA to its major catabolite, oxIAA and the two major, non-reversible, IAA conjugates IAAsp and IAGlu may play vital roles in inactivating the IAA built up by transport and biosynthesis, in order to maintain appropriate spatio-temporal levels of the hormone. It is also clear that IAA biosynthesis, signalling and degradation is regulated in a complex way, where auxin - cytokinin interactions play an important role. The

possibility remains that other hormones are also involved in this cross-regulation.

There are some basic technical questions that recur from papers I, II and III. How and when should samples be taken? Which tissues and what amounts of tissues should be sampled? This is particularly relevant when very small amounts of tissue are sampled.

What general conclusions can be drawn on a complex biological system from simple data sets where only one or a few metabolites have been quantified? To what extent can data from different model systems and organisms be compared? These questions should be considered every time a new experiment is being designed.

5 Cell Type Specific 'OMICS

5.1 Introduction

Systems biology can be defined in many ways. It can include many different analytical methods and fields of the biological sciences. Due to the complexity of the data and the frequently large datasets, specialised bioinformatics and mathematical modelling are also commonly used. Here I have combined Fluorescent Activated Cell Sorting with metabolomic, proteomic and transcriptomic analysis. One of the aims was to develop methods for future comparison of root hair (trichoblast) and non-hair (atrichoblast) cells on a cellular level.

Hair and non-hair cells are an excellent model system for looking at single differentiated cells, as they are easy to identify and isolate. Root hairs are involved in many important physiological mechanisms, such as nutrient and water uptake, and interactions with beneficial and pathogenic soil microorganisms (reviewed by Libault et al., 2010). These cell types are also interesting from an auxin perspective as normal root hair initiation and elongation requires auxin (Pitts et al., 1998). Auxin is involved in establishing root hair polarity (Grebe, 2004). According to Jones et al. (2009) non-hair cells should have approximately 10 times higher IAA concentration than hair cells. This has, however, not been confirmed experimentally. Two GFP expressing lines were used for the analysis of single cell transcriptomics, metabolomics and proteomics. For the trichoblasts, the COBRA LIKE9::GFP (COBL9::GFP) line was used and for atrichoblasts the GLABRA2::GFP (GL2::GFP) line was used (Figure 6 A,B).

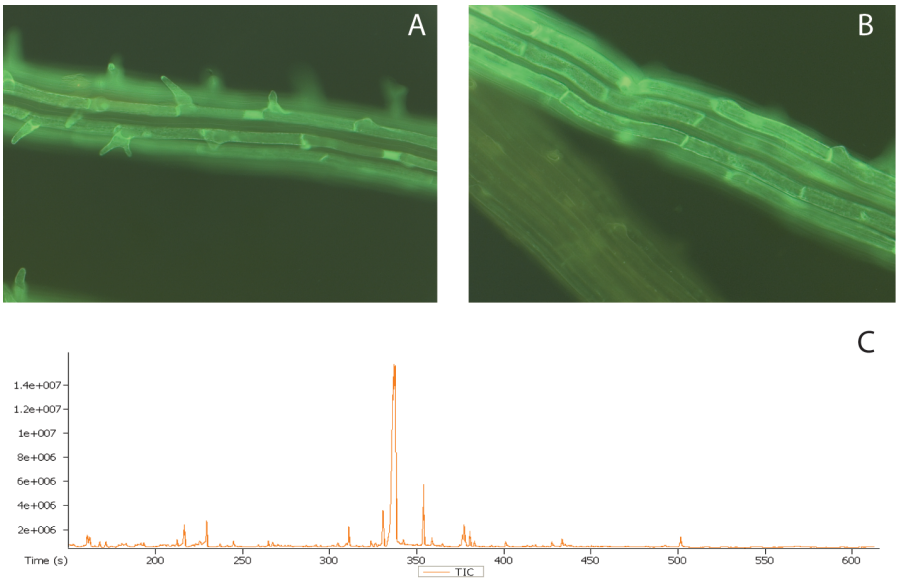

Figure 6. (A) The COBL9 line with root hair forming cells expressing GFP (B) and the GL2 line with non-hair forming cells expressing GFP. (C) Total Ion Chromatogram showing typical GC-TOFMS analysis of a protoplast sample.

Plants are morphologically simple organisms compared to higher animals and are considered to have approximately 40 different cell types. Each of the cell types are likely to be different, with regards to their transcriptome, metabolite and protein composition, and to be highly specialised in terms of the specific biological functions they carry out (Goldberg, 1988). The combination of relatively few, but specific, cell types makes plants ideal model systems for cell type or tissue specific 'omics analysis. Applying a cell type specific 'omics approach to the study of plant development can help in the identification of connections between pathways, and for the construction of models for developmental processes and networks. The main advantage is to eliminate the background noise that comes from studying mixed populations of cells that normally arises when studying whole plants or tissues. The cell-type specific analysis can also facilitate the identification of rare and low abundant metabolites and proteins (Moco et al., 2009).

The major challenge is to collect pure tissue samples in an accurate, robust, rapid and reproducible way. It can be challenging to dissect very small amounts of tissue. When collecting apical root sections, for example, the collection is undertaken on a moist agar plate in order to keep the root from drying out. This introduces the problem of moisture on the root

surface affecting the final sample weight. Drying the sample, for example by freeze-drying, or removing surface moisture by wiping with a tissue, will only introduce other forms of stress that can influence the final result. Another difficulty with root tip analysis is that the smaller the root sections are cut, the bigger will be the variation in weight. This problem is exacerbated because it is impractical to accurately weigh the material. It is also not practical to be more accurate in dissecting the tissue than the width of the tip of the scalpel blade. Cutting 0,5-1 mm long root sections, as was carried out in paper II, appears to be the limit of precision using manual techniques. There are other techniques for collecting tissue specific samples, such as mechanical micro-dissection, that has been used for cell type specific RNA extraction (Thome et al., 2006) or laser micro-dissection that can also be used to sample single cells (Moco et al., 2009). One of the drawbacks of these techniques is that they require cryosectioning or paraffin embedding prior to dissection and it is more difficult to obtain well-defined and sizeable cell populations.

Using FACS enables rapid, compared to micro dissection, collection of well-defined live cell populations, irrespective of their location in the root. Collecting a sample of GFP expressing protoplasts by FACS minimises ambiguity in the sample size as it is possible with FACS to determine exactly how much sample has been collected, down to the last cell. The goal of this work was therefore to enable accurate 'omics analyses by using sorted cells directly in the 'omics projects. Using this approach, the normalisation procedures could be simplified, allowing a more robust comparison of results between the biological and technical replicates.

The principal technical innovation was to apply published and well-established protocols for metabolomics, proteomics and transcriptomics on cells sorted by FACS to obtain cell-specific resolution. Whereas isolating samples of approximately 150,000 protoplasts / ml buffer for analysis was straightforward, the subsequent analyses presented a series of significant challenges.

5.1.1 Metabolomics

Plant metabolomics is a useful diagnostic tool for profiling metabolite composition of different genotypes, assessing natural metabolite differences between plant individuals and for functional annotation of genes (Schauer & Fernie, 2006). It is estimated that there is almost 200,000 metabolites in the entire plant kingdom the plant kingdom (Fiehn et al., 2002), with about 5,000-25,000 for different species of plants. There are many technical and analytical challenges in plant metabolomics such as the diversity of

compounds, the dynamic range of the metabolites, the way the metabolite composition changes over the lifespan of a plant and the spatial distribution of metabolites in different cell types and organelles (Hegeman, 2010). Applying a cell sorting approach to metabolomics can help to solve the problem of spatial distribution in an elegant way.

The main concern in metabolomics is to rapidly stop all metabolic processes, to make sure that the chemical identity of the metabolites are preserved and that it is possible to re-dissolve the sample again after extraction (Hegeman, 2010). Freezing the sample is the best way to stop all reactions. Freeze drying has also been suggested but is difficult as the sample becomes very hygroscopic and can resume enzyme activity if only a minute amount of moisture is reintroduced to the sample post drying (Fiehn, 2002). Sample collection is the weak point when pursuing single cell metabolomics with FACS as it takes a long time to enzymatically degrade the cell walls compared to simply harvesting a leaf and freezing it directly in liquid nitrogen.

Method development for protoplast metabolomics

Feeding roots with IAA and analysing the metabolite composition of trichoblasts and atrichoblasts should theoretically allow the identification of an important subset of data, and give a good indication of which metabolic pathways are involved in IAA responses in these cell types.
In the initial experiment, ~200,000 protoplasts (the same quantity that was used for the analysis of IAA levels and biosynthesis rates in previous experiments) were collected in FACS sheet buffer according to Petersson et al. (2009). The protoplasts were extracted and analysed according to Jonson et al. (2005) for their metabolic composition. The Jonson et al. (2005) protocol is developed for intact plant tissues e.g. leaves, and not cell or protoplast suspensions.

Except for a mannitol peak originating from the protoplast isolation buffer, the initial assays based on Jonson et al. (2005) did not detect any identifiable metabolites. It was decided that one of the main problems was that the sample size was too small for the subsequent procedures. Also, although mannitol stabilizes the osmotic potential of the solution and keeps the protoplasts intact, for metabolomic analysis, where all metabolites are interesting and therefore no purification is carried out, the presence of comparatively large concentration of mannitol makes it impossible to extract any metabolite data. Rinsing the cells before FACS to reduce the mannitol content did not make a significant difference. Spinning down the protoplasts and discarding the buffer was initially considered not to be a viable option,

because of the risk of uncontrollable loss of sample. Possible alternatives such as the evaporation or freeze-drying of the samples were tried. Removing the sorting buffer by evaporation at room temperature in a Speedvac took several hours and was quickly abandoned because of the detrimental effect this long process would have on the samples.

As the samples always should be kept frozen to minimize metabolic activity, freeze-drying was believed to be the best option. This proved to be technically possible, but the remaining powder was very electrostatic and proved difficult to handle. Furthermore, it was not possible to fully re-dissolve the powder in the extraction mixture. The powder obtained after freeze-drying that could be dissolved and analysed turned out to be high in phosphates. Phosphates interfere with the GC-TOFMS analysis, giving a large saturated phosphate peak that covered a third of the chromatogram, making it impossible to extract any useful data.

The FACS Flow™ buffer worked well for the GC-MS-derived IAA analyses, most likely because a post-FACS solid phase extraction (SPE) purification step was included in the process. Since the FACS buffer contains phosphates it was necessary to switch to a buffer that did not interfere with the metabolomics analysis. The phosphate-free MES buffer was initially tried unsuccessfully. It caused similar problems of precipitation to the FACS Flow™.

After the failure of the MES buffer, it was decided that the Johnsson et al. (2005) extraction protocol should be modified by the addition of a purification step and that a 0.7% NaCl solution should be used as the flow buffer. Internal standards for the metabolomics extraction (Salicylic acid, Myristic acid, hexadecanoic acid and cholesterol, proline, succinic acid, glutamic acid, alpha ketoglutarate, putrescine, glucose and sucrose) were mixed with a 0.7% NaCl solution as mock samples and several SPE columns were tested. However, none of the SPE columns could retain all the internal standards and so a combination of columns would have to be used in order for this approach to be successful.

Based on experience from the parallel proteomics work, which was similarly challenging because of Bovine Serum Albumin and phosphates in the sorting buffers, the sorted protoplasts were spun down and the supernatant was removed prior to analysis. To minimise problems associated with sample loss during this process, much larger sample sizes, 1-3 million protoplasts, were used. This approach gave the first set of samples that could be analysed (figure 6C). As collecting protoplasts by FACS is a considerable bottleneck in the procedure, it would have been beneficial if the samples could have been used for both metabolite and protein analyses. An attempt

was made to precipitate proteins from the samples after the metabolite extract procedure, however, whereas a precipitate did form, SDS gel analysis of the precipitate indicated that it was not proteins. This will, however, still be the aspiration in the future.

Data generated by protoplast metabolomics

After extraction, the samples were analysed by GC-TOFMS (Leco Pegasus II GC-TOF mass spectrometer; Leco, St. Joseph, MI, USA) and the data was processed and further analysed using the Leco software. The data was compared to in-house libraries (Table 1). Whereas this protocol enabled an analysis of metabolomic data, it had reintroduced the problem of uncontrolled loss of protoplasts and therefore an inability to determine precisely how many protoplasts were used for the analysis. The assumption was made, however, that for normalisation purposes the loss of material was equal for all samples.

The identified metabolites presented in Table 1 serve as a proof of principle and not a comprehensive metabolite profile. The list, from the samples containing a mixture of protoplasts from the root, comprehends flavonoids, fatty acids, sterols, sugars and organic acids.

Several examples of single cell or cell type specific metabolomics using plant cells have been published recently. The Arabidopsis trichome metabolome was analysed by Ebert et al. (2010), where they manually removed the trichomes from the surface of the leaf by scalpel blade. The major advantage of this method is that it was comparatively rapid from intact plant to extraction. The major drawback is that it would be difficult to extend this method beyond cell types that can be easily harvested because they protrude from the surface of the tissue. *In situ* Laser Ablation Electro Spray Ionisation-MS (LAESI-MS) has been used to obtain single cell metabolic data from epidermal cells of onion and daffodil (Shrestha & Vertes, 2009). This is a true single cell analysis process. The main drawback is, however, that it requires many more samples to obtain a representative picture of the epidermis, and it is therefore very time consuming and labour intensive. LAESI-MS also has works best for cells on the plant surface. Laser micro dissection has also been used to compare the metabolome of vascular bundles and surrounding tissues in Arabidopsis stem sections (Schad et al., 2005). In this analysis, plant tissue equivalent to about 5000 cells was dissected and analysed. Sixty-eight metabolites were identified, and the metabolite profile of vascular bundles and surrounding tissues could be separated by a principal component analysis. For obtaining a cell type specific metabolite profile from an internal tissue, however, FACS, coupled

with MS, is currently the best option. Once the various difficulties that remain with the FACS-based method have been solved, the next step will be to collect and analyse many different cell types in order to determine if it is possible to establish which metabolites that are specific to a certain cell type, similar to the original work of Schad et al. (2005). Optimising the protocols would also enable the determination of the effects that increases in IAA has on the metabolome of different cell types.

Table1. Identified metabolites in protoplasts isolated from Arabidopsis

2-oxovaleric acid	Isoleucine
24-methylcholesterol	Lactic acid
4-hydroxybenzoic acid	Lactose
Apigenin	Linoleic acid
Arabitol	Maltose
β-alanine	Nicotinic acid
Citric acid	Oxalic acid
Eicosanoic acid	Pyroglutamic acid
Elaidic acid	Rhamnose
Fructose	Salicin
GABA	Sebacic acid
Galactose	Sorbitol
γ-linolenic acid	Sorbitol-6-phosphate
Gluconic acid lactone	Stigmasterol
Glutamic acid	Xylose
Glutaric acid	Xylulos

The metabolites were identified in samples containing between 1.5-2.7 million Arabidopsis root protoplasts. Identification was made based on spectra recognition but without the use of a retention index.

Increasing resolution and identifying more metabolites will be crucial if single cell metabolomics by FACS will be able to add a new dimension to plant systems biology. One way to achieve this would be to fraction the protoplast sample and focus on groups of compounds, thereby increasing resolution. Reintroducing an SPE purification step may be a good alternative. Switching analytical methods from GC-TOFMS to LC-MS should also produce a different set of metabolites. GC-MS is a suitable method for compounds in the primary metabolism i.e. amino acids, fatty acids, carbohydrates and organic acids (Gullberg et al., 2004) and LC-MS is a good analytical technique for analysing secondary metabolites like alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates and polyamines (De Vos et al., 2007). The drawback of using

LC-MS is the lack of established spectra databases and the spectra generated by LC-MS vary slightly from one instrument to another making it difficult to build libraries similar to those available for GC-MS data (Hegeman, 2010). It is also difficult to obtain consistent quantitative precisions (Kaddurah-Daouk, et al., 2008).

5.1.2 Proteomics

Similarly to the objective for metabolomics, the initial aim of the proteomics work was to establish protocols for the extraction of the samples in the flow buffer in order to minimise problems with loss of material.
In the initial assays, the main problem was that the samples were contaminated by the protein, Bovine Serum Albumin (BSA), since the BSA quenched the signal from the lower abundance endogenous proteins. The protoplast isolation buffer published by Birnbaum et al. (2005) contains (BSA) to stabilise the activity of the enzymes, cellulase and pectolyase. Washing the cells to remove BSA was not feasible, as BSA appeared to adhere to the protoplast surfaces. It was necessary to switch to a BSA-free buffer. As it had been observed previously that the FACS buffer could interfere with subsequent analysis, assays were conducted to determine whether the buffer also affected the efficiency of protein extraction. A buffer of 0.7% NaCl was shown to be the most compatible with subsequent protein extraction protocols.

Once the BSA free protoplast isolation method had been established, and sorting was performed in 0.7 % NaCl, the sorted protoplast samples were concentrated from 2-4 ml (approximately 100,000 -300,000 protoplasts) to 500 µl on 10K Amicon filters (15ml). The concentrated protoplast sample was then lysed by repeated freezing and thawing. Total protein digestion was conducted as described by Bylesjö et al. (2009) and the resulting peptides were fractionated by offline Strong Cation-Exchange (SCX) column into 15 fractions. Each fraction was separately analyzed by reverse-phase LC-ESI-MS, using a nanoACQUITY ultra-performance liquid chromatography (UPLC) system coupled to a Q-TOF mass spectrometer (Q-TOF Ultima; Waters Corp.) as described by Bylesjö et al. (2009). Using this approach it was possible to identify over 150 proteins, however, the amount of sample was still a limiting factor.

To solve this, 500,000 protoplasts were sorted from trichoblasts and atrichoblasts (figure 6A,B) populations for a subsequent assay. These cells were centrifuged to remove the 0.7% NaCl buffer and the pellet was re-suspended in SDS-PAGE loading buffer for separation on SDS-gel. It was vital to reduce the amount of buffer, as the whole protoplast sample was

loaded on the SDS gel. In total 50 gel bands were excised from the SDS gel from the trichoblast and atrichoblast samples. The gel bands were then digested in-gel as described by Srivastava et al. (2009).

In this first full-scale analysis of trichoblast and atrichoblast protoplasts, 567 proteins that were common to both types of cells were identified. Two hundred and fifty eight proteins that were unique to the trichoblasts and 147 that were unique to atrichoblasts were also identified. Thirteen genes corresponding to identified proteins have been published by Yadav et al. (2009) as being up-regulated by the protoplast isolation process. Eleven proteins corresponding to auxin related genes were identified, three of them unique for trichoblasts and two unique to atrichoblasts (figure 7).

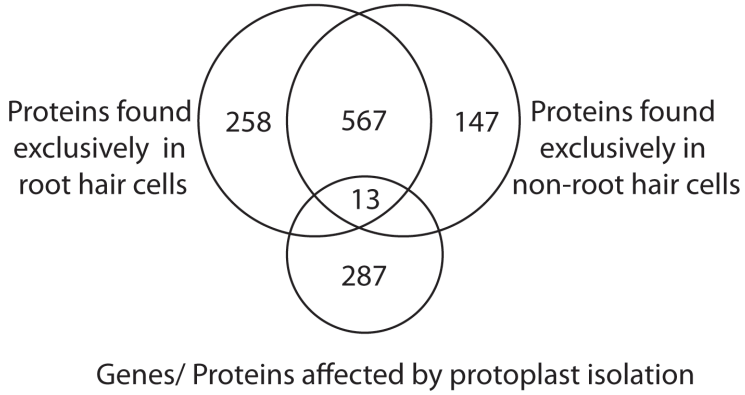

Figure 7. In Total 972 proteins were identified in the hair and non-hair cells by LC-QTOFMS.

This proteomics approach for studying IAA dependent developmental events will be continued. The next step will be to repeat the assay using several replicates of trichoblasts, atrichoblasts and reference cells to establish the peptide patterns for different tissues under normal conditions. Once this is well established, the effects of IAA on trichoblast and atrichoblast development will be examined.

5.1.3 Transcriptomics

In a collaboration with the lab of Stefan Kepinski, University of Leeds, trichoblast and atrichoblast cells were also sorted by FACS for transcriptional profiling to search for IAA related genes differentially expressed in hair and non-hair cells. Only the meristematic zone was used and because of non-specific GFP expression, the root cap had to be removed and the 500 μm long meristematic zone was dissected prior to protoplast isolation. Given

that this was a difficult and lengthy procedure, it was decided to dissect roots for only one hour to minimize stress-induced changes in the transcriptome. The RNA was extracted from the protoplasts with a Qiagen RNeasy kit. The RNA yield from 5000-10000 protoplasts was approximately 6 ng. The RNA was amplified by the Affymetrix Two-Cycle kit and the transcriptional profiling was done at the Arabidopsis stock centre in Nottingham.

Transcript profiling detected five IAA signalling related transcripts that were significantly up-regulated in the trichoblasts and eleven that were significantly up-regulated in the atrichoblasts.

Comprehensive transcriptional profiling using sorted Arabidopsis root cells was published in the ground-breaking papers by Birnbaum et al., 2003 and Brady et al., 2007. For the first time, these papers showed transcriptional profiles for specific plant cell types that had been obtained using FACS combined with microarray analysis. The assay used in our experiment was similar, although our aim was to characterize a biological event induced by IAA. One initial concern was the amount of protoplasts that we were able to be sorted. The aim of our experiments was to obtain 5000 protoplasts in order to be able to extract enough RNA without having to do too many rounds of amplification. Neither in Birnbaum et al., 2003, 2005 nor Brady et al., 2007 is the number of protoplasts used for RNA extraction for the array analysis declared. In the protocol from Birnbaum et al. (2005) they say that 300,000 GFP positive protoplasts per pooled biological can be used, but as little as a few thousand is enough. They also state that for rare cell types with a low yield, 100-500 protoplasts, amplification is recommended. Most of the GAL4-GFP lines used in our study were considered low yielding lines, where the sorted population constituted only ~1-5% of the events in the gated population. According to the original protocol published by Birnbaum et al (2005), the sorting should not extend to more than 60 min because of transcriptional changes. Sorting enough material for IAA analysis, metabolomics and proteomics within this time limit is technically impracticable, almost impossible. As the total protoplast isolation, from intact roots to the last sorted protoplast, takes at least 2.5 hours, depending on the sorting speed and efficiency, transcriptional changes are inevitable. The protoplasts are kept in the dark at 4°C during sorting, minimizing cellular activity, but kept in daylight at room temperature during the isolation process. Therefore, it is the time required for protoplast isolation that needs to be most carefully controlled and limited. The temperature optima for the cell wall degrading pectolyase and cellulase enzymes are ~35°C, but this is not a suitable isolation temperature even if it the isolation

would be faster, as it might induce temperature and other abiotic stress induced metabolic changes.

Also, there is no doubt that global gene expression will be perturbed when the intercellular communication and cell walls are lost during protoplasting (Galbraith, 2003). Data show that ~300 genes responded significantly to the protoplasting process itself (Yadav et al., 2009). Many of these genes are involved in IAA metabolism and signalling. Together, this indicates that great care should be taken when interpreting transcriptional profiling data.

5.2 Summary – OMICS

The aim of the 'omics work was to develop methods that can be used for obtaining a systems biology understanding of the cellular processes that determine the IAA dependent developmental changes in particular cell types. Combining metabolic, proteomic and transcriptomic data from specific cell types, i.e. in trichoblasts and atrichoblasts under normal conditions will help us with a basic understanding of the root hair biology. Once established, the changes induced by IAA treatment will be examined. The main obstacles for successfully conducting these experiments are currently the lack of suitable GFP lines with precise expression patterns, and the process of collecting the samples, which is slow and places stresses on the protoplasts. These stressful conditions are expected to lead to changes in cellular function, which are difficult to remove by normalisation. One by one, however, it is expected that each of these difficulties will be able to be overcome. Once they have, the aim is to eventually perform IAA treatments to conduct systems biology 'omics studies of the processes dependent on this hormone, on a cell-type specific basis.

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