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Cytokinins in Higher Plants: Biosynthesis and interaction with auxins

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Abstract

Cytokinin biosynthesis and cytokinin/auxin interactions were studied in wildtype and hormone-overproducing plants. To enable the physiological studies to be performed, analytical tools based on liquid chromatography/ mass spectrometry (LC/MS) were developed, allowing cytokinins in minute amounts of plant tissue to be analysed.

A method for *in-vivo* deuterium labelling of cytokinins provided evidence for the existence of an alternative, iPAMP-independent pathway for cytokinin biosynthesis in plants. The alternative pathway was active both in wildtype plants and in plants expressing the isopentenyltransferase (*ipt*)-gene from *Agrobacterium tumefaciens*. In the *ipt*-expressing, cytokinin overproducing plants, the rate of ZRMP production was found to be 66 times higher than that of iPAMP formation. This indicates that iPAMP is not the major precursor of ZRMP in the bacterial pathway.

IAA biosynthesis was also studied, in tobacco, to determine the relative importance of tryptophan-dependent and tryptopan-independent biosynthesis. From tracer studies, it was concluded that the independent pathway contributed the majority of the IAA synthesised during vegetative growth.

In another series of experiments, cytokinin/auxin interactions in hormone-overproducing plants were studied. Cytokinin overproduction was shown to decrease the rate of auxin biosynthesis, resulting in lower levels of IAA. Likewise, lower cytokinin levels were found in plants that overproduced auxins. In order to investigate the interactions further, cytokinin and auxin overproducing plants were crossed. The offspring expressed all the phenotypic traits associated with both of the parental lines, giving rise to a mixed phenotype. Surprisingly, did not the hormone levels of the cross significantly differ from wildtype plants. Thus, were the increased hormone levels of the parental lines downregulated in the cross but the plant line did still express the phenotypical traits associated with auxin- and cytokinin-overproduction.

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ABSTRACT

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Cytokinin biosynthesis and cytokinin/auxin interactions were studied in wildtype and hormone-overproducing plants. To enable the physiological studies to be performed, analytical tools based on liquid chromatography/mass spectrometry (LC/MS) were developed, allowing cytokinins in minute amounts of plant tissue to be analysed. A method for *in-vivo* deuterium labelling of cytokinins provided evidence for the existence of an alternative, iPAMP-independent pathway for cytokinin biosynthesis in plants. The alternative pathway was active both in wildtype plants and in plants expressing the isopentenyltransferase (*ipt*)-gene from *Agrobacterium tumefaciens*. In the *ipt*-expressing, cytokinin overproducing plants, the rate of ZRMP production was found to be 66 times higher than that of iPAMP formation. This indicates that iPAMP is not the major precursor of ZRMP in the bacterial pathway. IAA biosynthesis was also studied, in tobacco, to determine the relative importance of tryptophan-dependent and tryptopan-independent biosynthesis. From tracer studies, it was concluded that the independent pathway contributed the majority of the IAA synthesised during vegetative growth. In another series of experiments, cytokinin/auxin interactions in hormone-overproducing plants were studied. Cytokinin overproduction was shown to decrease the rate of auxin biosynthesis, resulting in lower levels of IAA. Likewise, lower cytokinin levels were found in plants that overproduced auxins. In order to investigate the interactions further, cytokinin and auxin overproducing plants were crossed. The offspring expressed all the phenotypic traits associated with both of the parental lines, giving rise to a mixed phenotype. Surprisingly, did not the hormone levels of the cross significantly differ from wildtype plants. Thus, were the increased hormone levels of the parental lines downregulated in the cross but the plant line did still express the phenotypical traits associated with auxin- and cytokinin-overproduction.

Keywords: cytokinin, auxin, biosynthesis, isopentenyltransferase, transgenic plants, Arbidopsis thaliana, Nicotiana tabacum, Agrobacterium tumefaciens, LC-MS, deuterium oxide, *in-vivo* labelling.

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CONTENTS

APPENDIX

List of papers

The present thesis is based on the following papers, which will be referred to by the corresponding Roman numerals.

I. **Åstot, C., Dolezal, K., Moritz, T. and Sandberg, G.** (1998). Precolumn Derivatization and Capillary Liquid Chromatographic / Frit-Fast Atom Bombardment Mass Spectrometric Analysis of Cytokinins in *Arabidopsis thaliana*. *Journal of Mass Spectrometry* 33: 892-902.

II. **Åstot, C., Dolezal, K., Moritz, T. and Sandberg, G.** (2000). Deuterium *In Vivo* Labelling of Cytokinins in *Arabidopsis thaliana* Analysed by Capillary Liquid Chromatography / Frit-Fast Atom Bombardment Mass Spectrometry. *Journal of Mass Spectrometry* 35: 13-22.

III. **Åstot, C., Dolezal, K., Wang, Q., Kunkel, T., Moritz, T., Chua, N.-H. and Sandberg, G.** (2000). An Alternative Cytokinin Biosynthesis Pathway. (Submitted).

IV. **Eklöf, S., Åstot, C., Moritz, T., Blackwell, J., Olsson, O. and Sandberg, G.** (1996). Cytokinin Metabolites and Gradients in Wild Type and Transgenic Tobacco with Moderate Cytokinin Over-production. *Physiologia Plantarum* 98: 333-344.

V. **Eklöf, S., Åstot, C., Moritz, T., Blackwell, J., Olsson, O. and Sandberg, G.** (1997). Auxin-Cytokinin Interactions in Wild-Type and Transgenic Tobacco. *Plant and Cell Physiology* 38(3): 225-235.

VI. **Sitbon*, F., Åstot*, C., Edlund, A., Crozier, A and Sandberg, G.** (2000). The relative importance of trypthophan-dependent and tryptophan-independent biosynthesis of indole-3-acetic acid in tobacco during vegetative growth. *Planta* (Accepted for publication).

VII. **Eklöf*, S., Åstot*, C., Moritz, T., Sitbon, F., Olsson, O. and Sandberg, G.** (2000). Transgenic Tobacco Plants Co-Expressing *Agrobacterium iaa* and *ipt* Genes have Wildtype Hormone Levels but Display Both Auxin and Cytokinin-Overproducing Phenotypes. *Plant Journal* (Accepted for publication).

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BACKGROUND

1. Introduction

Multicellular organisms are dependent on signalling systems to control and integrate cellular responses involved in growth and development. This integration is partly mediated by hormones, biologically active chemical substances that affect specific physiological processes in target organs at very low concentrations (<1 μM).

In animals, the hormones are normally synthesised in endocrinal glands, secreted into the blood stream and transported to target organs, often located a long distance from the site of synthesis. In plants, hormones can also be transported long distances, in the "transpiration stream" of the xylem, in the phloem sap or, in the case of auxin, by polar transport in the vascular cambium. However, endocrine concepts do not fully apply to plants since examples of hormones synthesised close to the site of action are also found. One such example involves ethylene and fruit ripening, since ethylene almost certainly promotes ripening of the very cell that synthesises it.

There are five classes of "classical" plant hormones; auxins, cytokinins, ethylene, abscisic acid and giberellins, which alone or together affect various processes in plant growth and development. New classes of substances, such as brassinosteroids and jasmonic acid, have recently been identified as regulators of plant development and yet others may remain to be identified. But among the known hormones, auxins and cytokinins remain the two most important classes of endogenous substances regulating growth and development in plants.

2. Auxins

Auxin was first described as the compound(s) that causes curvature of oat coleoptiles toward light (Went 1928). The most important auxin was later identified as indole-3-acetic acid (IAA) (Thimann and Koepfli 1935; Igochi *et al.* 1971) followed by later identifications of other endogenous substances with similar activity (Wightman and Lighty 1982; Erntsen and Sandberg 1986; Ludwig-Müller and Epstein 1991). IAA exists not only as the free, presumably active, form but also as sugar and aminoacid conjugates that are important storage forms in, for example, seeds. The major sites of IAA-synthesis in plants are supposed to be the buds and the young, developing leaves, but no conclusive data exists to prove this. However, IAA is transported basipetally from the apical plant organs of dicotyledons at rates of around 1 cm h^{-1} through parenchyma cells of the vascular cambium (Lomax *et al.* 1995). The polar transport is an active process mediated via specific carriers in the parenchymatic cells surrounding the vascular strands (Galweiler *et al.* 1998).

IAA affects cell enlargement and cell division of plants, and it is involved in diverse developmental processes, such as apical dominance (Philips 1975; Tamas 1995), root initiation (Thimann and Koepfli 1935), differentiation of vascular tissue (Sachs 1981; Aloni 1995; Little and Pharis 1995) and tropic responses (Briggs 1963; Harrison 1989; Evans 1991).

3. Auxin Biosynthesis

The aminoacid tryptophan was earlier considered to be the single biosynthetic precursor of IAA. From tryptophan two or possibly three pathways to IAA exist in plants, involving indolepyruvic acid, indoleacetonitrile or tryptamine as intermediates (Nonhebel *et al.* 1993). A tryptophan-independent biosynthesis pathway has been proposed more recently, including an indolic tryptophan precursor, possibly indole-3-glycerol phosphate or indole, which acts as a branch point for IAA biosynthesis without prior conversion to tryptophan (Wright *et al.* 1991; Normanly *et al.* 1993). The tryptophan-independent pathway has been shown to contribute the major part of IAA biosynthesis in several plant species (Normanly 1997).

The endogenous IAA-biosynthesis genes are unknown except for nitrilase genes from *Arabidopsis thaliana* (Bartel and Fink 1994), involved in the indoleacetonitrile-pathway to IAA, which is considered a pathway specific to Brassicacae species. However, a few bacterial analogues to the plant biosynthesis genes have been identified (Koga *et al.* 1991; Costacurta *et al.* 1994). Two genes, *iaaM and iaaH*, cloned from the TDNA of the crown-gall inducing bacterium *Agrobacterium tumefaciens* encode an IAA biosynthetic route unique to microbes. The enzymes convert L-tryptophan to IAA with indoleacetamide as the intermediate. Transgenic expression of the *iaaH* and *iaaM* genes has become an important tool in plant science research to alter the endogenous IAA-content of various plants.

4. Cytokinins

Cytokinins are defined as substances that promote plant cell division in the presence of auxins. Kinetin or 6-furfurylaminopurine (Fig. 1a), the first cytokinin identified (Miller and Skoog 1955; Miller *et al.* 1956), is not an endogenous substance in plants but was derived from heated DNA. This finding prompted the search for structurally related cytokinins and later, Letham et al. (Letham 1963; Letham *et al.* 1964) identified the first endogenous plant cytokinin, *trans*-6-(4-hydroxy-3-methylbut-2-enylamino)-purine and the compound was named zeatin after the plant source; *Zea mays* (Fig. 1a). Today, adenine species substituted at N^6 with an isoprenoid or an aromatic sidechain remain the most important compounds with cytokinin activity found in plants (Fig. 1b) (Shaw 1994). There are also other substances with cytokinin activity, such as certain phenylurea derivatives (Shudo 1994), but only adenine cytokinins will be examined in this thesis.

Depending on the sidechain structure, the isoprenoid cytokinins are divided into the zeatin-, dihydrozeatin- and isopentenyl-classes (Fig. 1b) which all occur as free bases, nucleosides, nucleotides and glucosides (Fig. 1c and 2). The aromatic cytokinins are classified into the benzylaminopurine (BAP)-type and two types of hydroxylated BAP's (*ortho*- and *meta*-topolin). For convenience, some compounds that actually lack cytokinin activity are also termed "cytokinins" due to their structural similarities. Two stereoisomers exists, for example, of zeatin and its sugar conjugates; the (E)- or *trans*-isomers and the (Z)- or *cis*-isomers (Fig. 1b). All the *cis*-isomers lack biological activities but are still referred to as "cytokinins".

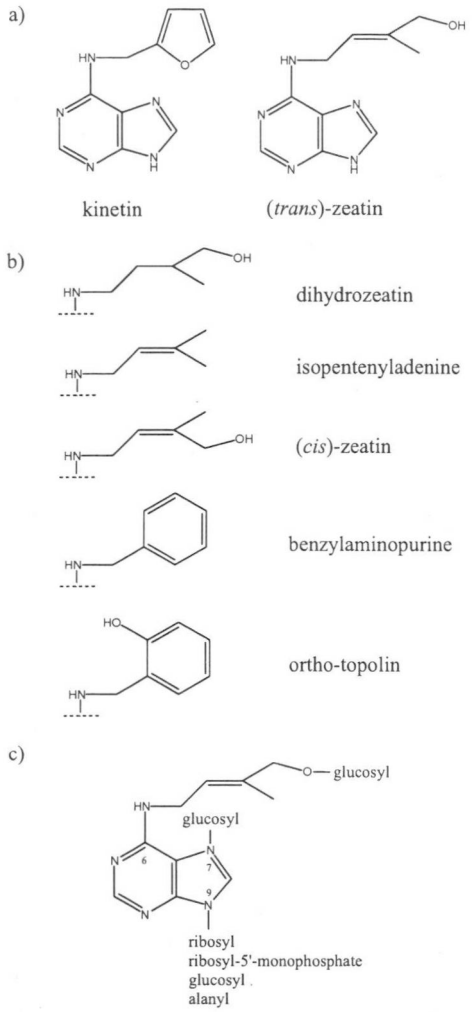

Fig. 1 Structure of adenine cytokinins.
a) Two cytokinin bases: kinetin, the synthetic cytokinin identified by Miller *et al.* (1956) and zeatin, a highly active plant endogenous cytokinin.
b) Other classes of isoprenoid (top three) and aromatic cytokinin bases (bottom two), defined by sidechain structure.
c) Conjugation of isoprenoid cytokinins. Conjugation sites in the purine ring (N-conjugates) or at the Z and DHZ sidechain (O-conjugates) are indicated. Important conjugation groups are exemplified (ribosyl = β-D-ribofuranosyl, glucosyl = β-D-glucopyranosyl, alanyl = L-alanyl). (See also table 1 in **I**).

Several different systems of cytokinin nomenclature are to be found in the literature. In this thesis, the isopentenyl-class of cytokinins are named as derivatives of adenine, adenosine and adenosine-5'-monophosphate. The nomenclature used for the zeatin- and dihydrozeatin-classes is based on the trivial names of the free base. Conjugation is indicated by adding the name of the conjugating group after the name of the free base.

Besides being factors regulating the plant cell cycle, cytokinins influence a wide array of important biological processes throughout the lifecycle of the plant (Mok 1994). These processes include seed germination (Khaan 1971), shoot bud formation (Skoog and Miller 1957) release of shoot buds from apical dominance (Wickson and Thimann 1958), leaf expansion (Kuraishi and Okumura 1956) and delay of senescence (Richmond and Lang 1957). It is however clear that plant development is determined by a number of internal and external factors and cytokinins are only one of these factors.

5. Cytokinin Biosynthesis and Metabolism

Cytokinins are formed both through "*de-novo*" biosynthesis and from degradation of transfer-RNA species that contain modified nucleotide bases such as isopententyladenine and *cis*-zeatin (Fig. 1b) (McGaw and Burch 1995). Initially the degradation of tRNA was considered the major source of free cytokinins, but calculations of tRNA turnover indicated that alternative pathways had to be present (Klämbt 1992). However, the first "*de-novo*" cytokinin biosynthetic enzyme was not isolated from a plant. It was identified in the cellular slime mould, *Dictyostelium discoideum*, in

which isopentenyladenine is significant as a precursor to discadenine, a spore germination inhibitor. It was shown that cell-free extracts of this organism were capable of converting dimethylallyl-pyrophospate (DMAPP) and adenosine-5'-monophosphate (5'-AMP) to the cytokinins isopentenyladenosine-5'-monophosphate (iPAMP) and isopentenyladenosine (iPA) (Taya *et al.* 1978). Based on this finding and current knowledge of the metabolism of isopentenyl-type cytokinins, a model for cytokinin biosynthesis was suggested, including iPAMP as the first cytokinin intermediate, with subsequent metabolism according to the scheme in Fig. 2 (Chen 1982; Letham and Palni 1983).

Later, a TDNA-encoded enzyme from the crown-gall inducing bacterium, *Agrobacterium tumefaciens*, was also shown to promote the transfer of an isopentenyl sidechain from DMAPP to 5'-AMP *in-vitro* and the enzyme was named isopentenyltransferase (IPT) according to this reaction (Akiyoshi *et al.* 1984; Barry *et al.* 1984). However, it was not possible to detect *in-vivo* formation of iPAMP in *ipt*-expressing crown-gall tissue (Stuchbury *et al.* 1979). Instead, formation of ZRMP was detected, and the possible existence of an alternative pathway, not involving iPAMP, was discussed. Later, however, the authors came to the conclusion that rapid conversion of iPAMP to ZRMP occurred (Palni and Horgan 1983) and the earlier result was re-evaluated (Palni *et al.* 1983). The alter-native pathway was never reviewed and the iPAMP-dependent cytokinin biosynthesis model became a paradigm (McGaw *et al.* 1984; McGaw 1988; Palni *et al.* 1990; McGaw and Burch 1995; Chen 1997; Prinsen *et al.* 1997). The findings prompted a search for endogenous plant cytokinin synthases related to the isopententyltransferase. However, no plant gene with sequence homology to the *ipt*-gene has been found (Morris 1988) and the endogenous cytokinin synthase(s) remains unidentified.

The major metabolic conversions of cytokinins found in plant material are indicated in figure 2. Cytokinin nucleotide-, nucleoside- and free base-interconversions are mediated by the same enzymes that convert their adenyl analogues, while the sidechain modifications are specific to isoprenoid cytokinins. They involve the stereospecific hydroxylation of the isopententyl sidechain to produce a *trans*-zeatin sidechain (Chen and Leisner 1984) and the reduction of a zeatin sidechain to produce a dihydrozeatin sidechain (Mok and Martin 1994) (dihydrozeatin species are not included in Fig. 2). Glucosylation of cytokinins in the N^7- or N^9-position of the purine ring is mediated by "cytokinin-glucosyltransferase", an enzyme specific for cytokinins (Entsch *et al.* 1979) and the enzyme mediating the formation of 9-alanine conjugates in lupin (*Lupinus angustifolius* L.) also show specificity to cytokinins (Letham and Palni 1983). The N-glucosides and N-alanine conjugates lack cytokinin activity in bioassays, indicating that their formation represents a permanent inactivation (Laloue 1977; Letham *et al.* 1983). Formation of O-glucosides is a common modification of zeatin and dihydrozeatin species (Letham *et al.* 1976) mediated by a cytokinin-O-glucosidase (Dixon *et al.* 1989). In contrast to the N-glucosides, these conjugates are not resistant to hydrolysis by β-glucosidases in plant tissues and O-glucosides are thus considered cytokinin storage forms (McGaw and Burch 1995).

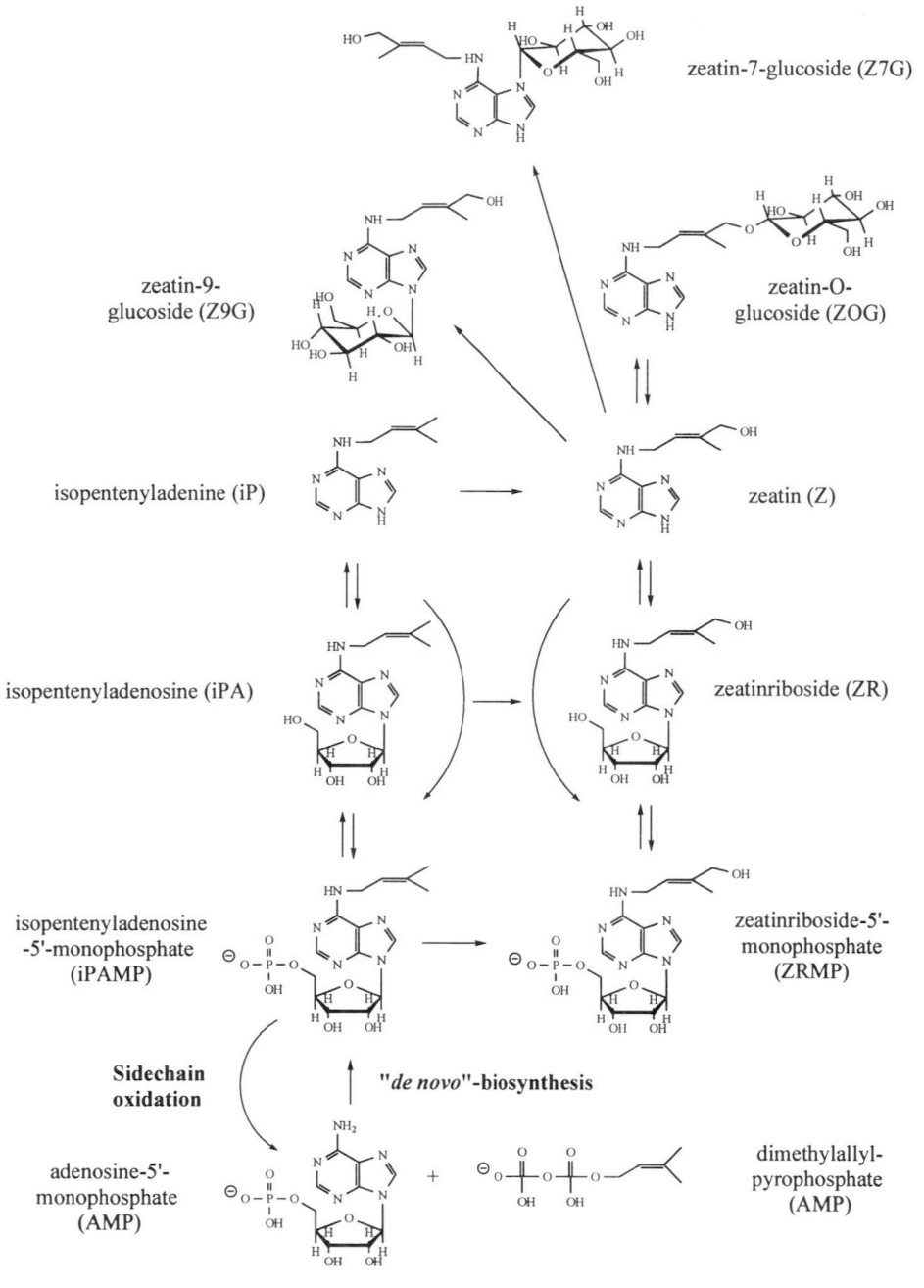

Fig. 2 Major metabolic pathways of isoprenoid cytokinins. *De-novo* biosynthesis is suggested to occurs at the nucleotide level with iPAMP as the first cytokinin intermediate. The nucleotides are converted to nucleosides and bases and zeatin is the proposed active compound. Three zeatin glucoside conjugates are formed (N^7- , N^9- and O-) and N-glucosidation also occurs for iP (not shown). Oxidative cleavage of cytokinin sidechain is exemplified for iPAMP but occurs also for the nucleosides and bases. (Dihydrozeatin cytokinin species not included in metabolism outline).

Permanent inactivation is also mediated by cytokinin oxidase, an enzyme that cleaves cytokinin sidechains from the adenine moiety with an oxidative reaction mechanism (reviewed by Hare and van Staden 1994)). Isopentenyl-type cytokinins have been shown to be the best substrates for the oxidase, the zeatin-type of cytokinins are converted at a slower rate and the dihydrozeatin-type cytokinins are not degraded by the oxidase. A correlation between the endogenous cytokinin content and the oxidase activity has been shown in *ipt*-transgenic plant tissues, and the importance of this relationship as a homeostatic mechanism has been emphasized (Zhang *et al.* 1995; Motyka *et al.* 1996). Recently the cytokinin oxidase was cloned (Houba-Herin *et al.* 1999; Morris *et al.* 1999) and activity was detected when the gene was expressed in moss protoplasts. A transit peptide has also been identified, and when the transformed moss protoplasts were cultured, the major activity was detected in the media. (Bilyeu *et al.* 1999). If extensive secretion of cytokinin oxidase also occurs *in planta*, apoplastic and symplastic cytokinin metabolism might differ significantly.

6. Cytokinin Transport and Sites of Biosynthesis

A large set of data indicates that roots and (more precisely) root tips, are major sites of endogenous plant cytokinin biosynthesis, but definitive evidence is still lacking (reviewed by Letham, 1994). The presence of ZR in root exudates supports the hypothesis (Purse *et al.* 1976) and the perceived importance of this flux in maintaining shoot growth has been stressed (Baker and Allen 1992). However, the capacity of detached shoots to grow independently of roots indicates that all rapidly dividing plant cells might have the capacity for cytokinin synthesis (Hewett and Wareing 1973). The significance of translocated cytokinins was recently questioned, based on experiments with tobacco grafts between rootstocks with inducible cytokinin-overproduction, and wildtype shoots. Although the roots contained 50-fold higher than wildtype ZR levels 24 h after induction, no increased cytokinin content in the shoots was detected, and a paracrine model of cytokinin action was presented (Faiss *et al.* 1997). The cytokinin profile of the transgenic tobacco line used in this experiment differs, however, from other cytokinin-overproducing plants in having a low ZRMP content. Assuming that the quantification data are not artefacts derived from faulty cytokinin analysis, the low nucleotide content might have an impact on the translocation capacity of the rootstocks.

Attempts to identify the sites of endogenous *de-novo* cytokinin biosynthesis using [^{14}C]adenine as a tracer have been performed (VanStaden and Forsyth 1984; Chen *et al.* 1985). However, the putative *de-novo* synthesized cytokinins were not unequivocally identified, and biosynthetic rates were calculated based on the radioactivity detected in compounds with chromatographic properties similar to cytokinin standards. The authors found incorporation rates of several percent after 12 and 21 days of incubation, respectively, and the significance of the results has been questioned (Letham 1994).

7. Cytokinin-Auxin Interactions

Cytokinins and auxins have been shown to interact and reciprocally modulate their mutual effects on a number of important developmental processes, such as organ formation from callus tissue (Skoog and Miller 1957) and apical dominance (Wickson and Thimann 1958). The variety of ways in which auxin and cytokinin regulate physiological responses (synergistic, antagonistic or additive) suggest that there may be multiple underlying mechanisms of interaction (reviewed by Coenen and Lomax 1997).

One potential mechanism of interaction is mutual control of the active auxin and cytokinin pool sizes by reciprocal action of the two hormones on each other's biosynthesis and/ or metabolism. Indications of this kind of mutual regulatory effect were detected, for instance, in early studies of plant tissues transformed with mutant TDNA from *Agrobacterium tumefaciens* (Akiyoski *et al.* 1983; McGaw *et al.* 1988). In cells transformed with TDNA containing mutagenized IAA-biosynthesis genes (*iaaM*, *iaaH*), producing inactive enzymes, dramatically higher cytokinin levels were found compared to crown-gall tissue expressing the wildtype TDNA, and if the cytokinin-biosynthesis gene (*ipt*) was mutated, disabling heterologous cytokinin production, a strong rise in the IAA-levels was found. Thus, mutual regulation is indicated but the mechanism is unknown. Auxin has also been shown to induce increased activity of cytokinin oxidase *in vitro*, thereby affecting the metabolic rate of cytokinins (Zhang *et al.* 1995). Regulation of transport may also have a role in modulating the active hormone pool size. For example, removal of the apical bud and thus the main IAA-source by decapitation of bean plants, *Phaseolus vulgaris,* has been found to cause a large increase in the cytokinin content of xylem exudates (Bangerth 1994; Li *et al.* 1995). The rise in the amount of transported cytokinins was eliminated by the application of NAA, a synthetic auxin, to the apex of the decapitated plants, supporting the significance of auxins in this response.

The classic experiments of Skoog and Miller (1957) on the growth of tobacco pith explant cultures demonstrated a synergistic action of cytokinins and auxins in the control of cell division. The cell cycle is mainly regulated at two "checkpoints", the G_1/S- and G_2/M- transitions, and progression through these control points is mediated by cyclin-dependent kinases (CDK's). To promote transition, a CDK has to be activated by the binding of specific cyclins, followed by phosphorylation. Genes encoding CDK's have been identified in several plant species and several classes exist (for recent review see Huntley 1999). The expression of the CDK-genes is, at least in part, transcriptionally regulated. Thus, it is interesting that auxin has been shown to induce cdc2-expression (Hirt *et al.* 1991; Martinez *et al.* 1992; Miao *et al.* 1993). Until recently, the role of cytokinins remained largely unknown, but links to the G2/M-transition was reported (Laureys *et al.* 1998; review in Hare and VanStaden, 1997), and cytokinins have been demonstrated to induce cdc2-expression in *Arabidopsis* roots (Hemerly *et al.*, 1993). A major breakthrough came when cytokinins were shown to induce expression of a D-type cyclin (Soni *et al.*, 1995) and, recently, cytokinin-induced cyclin D-

expression was reported to be important in the regulation of the G_1/S-transition in *Arabidopsis* (Riou-Khamlicki *et al.*, 1999). There are thus several points in the cell cycle that appear to be modulated by hormones, and the rapid progress in the field may provide additional cytokinin signalling elements in the future (D'Agostino and Kieber, 1999).

8. Objectives

Although cytokinins have been recognised as regulators of plant development for more than 30 years, many of the key questions concerning their biosynthesis, mechanism of action and homeostatic regulation, remain largely unanswered. The fact that cytokinins are present as endogenous plant compounds at very low concentrations, i.e. 1-100 pmol g^{-1} fresh weight, makes their detection and quantification technically demanding and early studies of cytokinin physiology were often impaired by inadequate methods of analysis.

The objective of the work presented in this thesis was to develop new tools to improve cytokinin characterisation of plant tissues. Mechanisms for cytokinin homeostasis were to be examined both in wildtype plants and in transgenic, cytokinin overproducing plant lines. A further goal was to study cytokinin/auxin interactions *in planta*, using cytokinin and auxin overproducing tobacco lines as model systems. The development of a tool for tracing cytokinin biosynthesis (monitoring deuterium enrichment kinetics) enabled a new type of experiment to be performed. Using this technology, we wanted to examine the pathways of cytokinin biosynthesis in both wildtype and *ipt*-expressing plant tissues.

Tobacco was used in early work in our laboratory due to its amenability towards *Agrobacterium*-mediated transformation. The construction of *ipt*-expressing lines was performed by Staffan Eklöf (1996) and the *iaaM*+*iaaH*-expressing lines were developed by Folke Sitbon (1992). For the later studies, *Arabidopsis thaliana* was chosen since it gave easy access to a system for inducible cytokinin–overproduction.

EXPERIMENTAL

9. General Aspects of Methodology

9.1 Analysis of cytokinins by mass spectrometry

The hyphenation of mass spectrometry and gas or liquid chromatography has greatly improved the analysis of organic molecules present at trace concentrations in complex biological matrices such as plant tissues (Newton and Walton 1996). In plant hormone research, work with gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) has provided very important qualitative and quantitative data (reviewed by Hedden 1993). A mass spectrometer is a highly selective detector, and compounds co-eluting from the chromatographic column are resolved in the mass analyser based on their mass to charge-ratios (m/z). Fragmentation of the analytes is normally induced, giving rise to a m/z spectrum with a number of specific ions. A substance from a plant extract is unequivocally identified when the mass spectrum obtained and the chromatographic retention time are identical to data acquired from the analysis of a synthetic standard. There are other analyti-

cal techniques, e.g. nuclear magnetic resonance (NMR), that are even more powerful for determining the structures of organic compounds, but these techniques generally suffer from lower sensitivity and a requirement for highly purified samples.

Analysis by GC-MS of polar substances with low volatility, e.g. cytokinins, is complicated by their high boiling points, which are significant restraints in gas chromatography. Conversion of such compounds to more volatile derivatives, suitable for gas chromatography, is thus a prerequisite. Cytokinins have mainly been analysed as their per(trimethylsilyl)- or permethyl-derivatives (Palni *et al.* 1986) but it has been a problem to find derivatization procedures that are robust and straight-forward. In recent years cytokinin analysis by LC-MS has gained importance (Hedden 1993; Imbault *et al.* 1993; Yang *et al.* 1993; Prinsen *et al.* 1995) (**I**) and the sensitivity obtained for cytokinins is generally higher than with GC-MS.

Prior to analysis by GC-MS or LC-MS, the bulk of the substances in the plant samples has to be removed. During any purification procedure, partial losses of the analytes are unavoidable. In quantitative analysis, compensation for the losses is essential to achieve reliable and correct data. The properties of mass spectrometry make it well suited to quantification by stable isotope dilution analysis (Rittenberg and Foster 1940) and the method has for a long time been used to quantify cytokinin content in plant tissues (Scott and Horgan 1980). The method is based on the principle that the loss during isolation of a stable isotope-labelled hormone standard (e.g. a ^{13}C-, ^{2}H- or ^{15}N-labelled analogue) added to the sample will be identical to the loss of the endogenous, unlabelled compound. The amount of unlabelled compound in the sample is derived from the known amount of labelled standard added and the ratio between unlabelled and labelled compounds acquired by the mass spectrometer. Method errors below 5% relative standard deviation are routinely obtained when quantification is performed according to the stable isotope-dilution procedure.

9.2 Metabolic analysis using tracers

When tracers (synthetic hormones or hormone precursors labelled by stable or radioactive isotopes) are fed to plant tissues, the metabolic conversions that follow are indicated by the detection of labelled metabolites. This methodology has brought invaluable information concerning biosynthetic and metabolic pathways. Radioactive tracers and high performance liquid chromatography / radio-counting (HPLC/RC) is a powerful tool when the metabolic fate of the tracer is unknown or when metabolic patterns are to be compared (**V**). Stable-isotope labelled tracers are preferred when the metabolites are to be identified by mass spectrometry (Östin *et al.* 1995). It is also important to verify the existence of the metabolites as endogenous substances. This is due to the fact that the metabolic fate of an exogenously supplied tracer compound may be different from the metabolism of the endogenous analogue. Artefacts may arise from altered metabolism induced by the feeding, abnormal distribution of the tracer or from effects of surgical manipulations used to bring the precursors into contact with the intracellular enzyme systems. In the plant cell, an enzyme and a substrate might be separated into dif-

ferent compartments, thereby preventing conversion *in-vivo*. If an exogenously supplied tracer diverges from the compartmentalization, an artificial conversion will be detected. A recent example is shown in the work by Rapparini *et al.* (1999). Here, exogenously supplied [$^2H_5$]tryptophan was found to be converted to [$^2H_5$]IAA at a higher rate than the conversion-rate of the endogenous Trp-pool, measured by *in-vivo* labelling through metabolic conversion of labelled anthranilate, a Trp-precursor. Thus, the importance of the tryptophan-dependent IAA-biosynthesis was overestimated when the analysis relied solely on [$^2H_5$]tryptophan-feeding.

Deuterium oxide (^2H_2O) is a tracer that lacks the drawbacks mentioned earlier. No surgical manipulations are needed since ^2H_2O has access to all compartments of the plant cell (Mitra *et al.* 1976). When plants are incubated in liquid media enriched with ^2H_2O, a general labelling process starts in essentially all metabolic pathways of the plant cell. Unlabelled compounds are replaced by deuterium labelled compounds at a rate dependent on the metabolic turnover and the precursor enrichment (i.e. the ratio of labelled- to unlabelled-precursor molecules). The biosynthesis of labelled compounds by the plant endogenous pathways has the advantage over other tracer experiments that the system will remain in steady state with a "normal" distribution of the tracer. This implies that the conversion of the tracer will essentially reflect the *in-vivo* metabolism.

Deuterium labelled substrates have, unfortunately, a slightly lower binding affinity to enzymes due to the "deuterium effect" (Katz *et al.* 1965) causing growth inhibition if the deuterium enrichment is high. When the deuterium content in the growth media is limited to 30%, for instance, no effect on general morphology and plant architecture is observed. A partial reduction of the growth rate is however found, e.g. rate of growth was reduced by 23% for *Zea mays* seedlings on 30% ^2H_2O -media (Pengelly and Bandurski 1983 and refs therein).

Despite the advantages mentioned above, *in-vivo* deuterium labelling can not completely replace other tracer studies. To provide evidence for a precursor-product relationship more specific tracers than ^2H_2O are needed since the deuterium label detected in a product could be derived from other, unknown precursors that will also be enriched by deuterium labelling during the experiment.

9.3 Cytokinin- and auxin- overproducing plants as model systems

The effects of hormones on plant morphology were originally investigated by exogenous application of synthetic substances with hormone activity. Results obtained by this approach are not always reproducible, due to variations in, for example, uptake and transport (Kaminek *et al.* 1997). The use of transgenic plants overproducing cytokinin or auxin by ectopic expression of the *Agrobacterium tumefaciens ipt*, or *iaaM* and *iaaH* genes, respectively, circumvents some of these problems. Data from such transgenic systems with constitutive expression have largely corroborated classical studies (Klee *et al.*, 1987; Smigocki and Owens, 1989; Ondrej *et al.* 1991; Yusibov *et al.*, 1991; Sitbon *et al.*, 1991, 1992). However, concerns have also been raised about the causes of some of the differences found between

transgenic and wild-type plants. Altered levels of potent growth regulators will probably have an effect on general metabolism in plant cells, and secondary effects may be associated with the hormone overexpression. Analysis of plant lines with weak or inducible expression of the bacterial genes is a possible way to avoid or reduce the "secondary effects" and may provide complementary information about the effects of hormone overproduction.

Another complication is that the altered development of plants overproducing hormones gives rise to problems in defining the developmental stage of the overproducing plant lines compared to wildtype plants. Should a hormone-overproducing plant be compared with wildtype plants of equal age, with an equal number of leaves, with an equal proportion of full height, or some other criterion? This problem highlights the importance of performing experiments at several ages and of comparing plants when the phenomena being observed are relatively stable within the plant lines.

RESULTS AND DISCUSSION

10. Development of LC-MS Methods for Cytokinin Analysis

10.1 Background

The problems associated with cytokinin analysis by GC-MS prompted the development of new LC-MS methods. In liquid chromatography, the polarity and low volatility of the cytokinins are not severe restraints, as they are in gas chromatography. A LC/frit-FAB-MS-method for cytokinins was previously developed in our lab (Imbault *et al.* 1993) and in this thesis further developments of this technology are described, aiming for an increased sensitivity and a higher sample throughput. We also tested an alternative LC/MS interfacing technique: electrospray ionization (ESI).

10.2 Electrospray ionization mass spectrometry: Principles of action

The electrospray technique is based on the formation of gas-phase ions when an aqueous liquid stream is pumped into a strong electrical field. The liquid flow normally enters the electrospray cell *via* a hollow needle, held at ground potential. A counter electrode with a potential of 2-5 kV is positioned at a few centimetres distance, and the high field strength promotes the formation of small, charged droplets. The solvent molecules are evaporated, and the droplets are shrunk, by drying the spray with a stream of heated gas. Charge repulsion increases in the shrinking droplets, and finally forces ions to evaporate from the droplets. When an ion sampling inlet of a mass spectrometer is positioned by the electrospray counter electrode, the ions can be transferred into the mass analyser. The electrospray ionization is a soft ionization technique generating protonated quasi-molecular ions with little or no fragmentation, and with a very low chemical background noise. In-source collision induced dissociation (CID) can also be used, to induce fragmentation and enrich the spectra with additional and more abundant fragment ions.

10.3 Cytokinin analysis by electrospray ionization mass spectrometry

The performance of an electrospray ionization interface (ESI) (Analytica of Bradford, Whitehouse-Fenn design)

coupled to a double focusing magnetic sector mass spectrometer of reversed geometry (Jeol JMS-SX102) was tested to assess whether the system could give the needed sensitivity. The in-source CID, ESI spectra of Z and ZR standard samples (Fig. 3a and b) are examples of ESI-data, showing the high signal/noise-ratios that were obtained. However, the stability of the interface in terms of ion transmission was poor, limiting the performance of the technique. Improved design of modern electrospray interfaces has solved the stability problems, and quantitative analysis of cytokinins by ESI-tandem mass spectrometry has been reported (Prinsen *et al.* 1995).

Nevertheless, the analyte signal from an electrospray interface is highly susceptible to suppression by other sample constituents, due to the physical principles of the electrospray. Thus, extensive purification, including immunoaffinity chromatography (IAC), is needed for cytokinin samples analysed by the ESI-tandem MS method (Van Onckelen, H. pers. comm. 1999). Therefore, cytokinins like Z7G and ZOG could not be analysed as intact molecules due to their lack of epitopes for antibodies raised against cytokinins. Conversion of ZOG to Z by β-glucosidase prior to purification by IAC enables estimation of the ZOG-content but Z7G is only hydrolysed by strongly acidic conditions, which are not suitable for samples containing deuterium-labelled internal standards. In a number of publications, quantification data on the Z9G-content acquired by the ESI-tandem mass spectrometry method, has been referred to as pooled "ZNG-content" (Prinsen *et al.* 1995; Motyka *et al.* 1996; Redig *et al.* 1996; Redig *et al.* 1997). Since the Z7G-content is expected to be included in the "ZNG-contents",

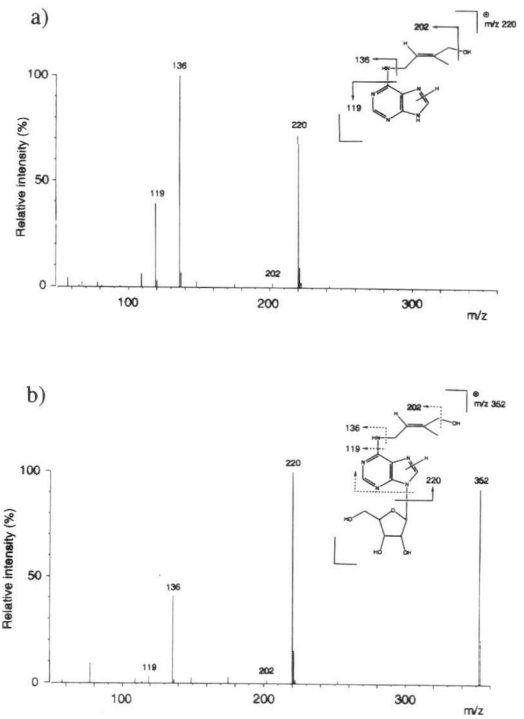

Fig. 3 ESI, in-source CID spectra of **a)** zeatin and **b)** zeatinriboside standard samples. Fragmentation pattern are indicated by full lines. Dotted lines corresponds to ions formed by multiple fragmentation including loss of sugar moiety.

erroneous conclusions concerning the importance of Z7G in wildtype and *ipt*-expressing plants might be drawn from this data.

10.4 Frit-FAB mass spectrometry: Principles of action

In the frit-fast atom bombardment (frit-FAB) ionisation technique, a liquid stream containing 1-10% glycerol is introduced through a porous steel frit to the vacuum region of a mass spectrometer's ion source (Ito *et al.* 1985). The liquid globule, formed on the steel frit, is bombarded with xenon atoms of high kinetic energy (4-8 kV).

Matrix molecules are ionized in the collision cascades (net ionization) and protonated matrix- and analyte-ions are formed by secondary ion-molecule reactions (Sunner 1993). The energy transferred by the collision cascade also drives the thermal desorption of charged and neutral species. The sensitivity of FAB-MS towards organic bases, such as the cytokinins, is generally improved if protonated ions are preformed by acidification of the matrix (Shiea and Sunner 1991). The effect is pronounced for bases with a high surface activity due to their accumulation on the surface of the globule, where the impact of the cascade is high (Ligon and Dorn 1986). Thus, for example, the FAB-MS sensitivity of the hydrophobic cytokinin nucleoside iPA is 20-fold higher than the sensitivity of ZR, a more polar nucleoside.

A major disadvantage of the FAB-ionization technique is the chemical noise the glycerol matrix gives rise to in analysis in normal scanning or low-resolution selected ion monitoring (LR-SIM) mode. The interference of the matrix can be decreased using measurement modes with higher selectivity, e.g. high-resolution SIM (Imbault *et al.* 1993), tandem mass spectrometry (Crow *et al.* 1984) or selective reaction monitoring (Moritz 1995) (**I**) (see 10.7).

10.5 New cytokinin derivatives for frit-FAB mass spectrometry

It is possible to increase the surface activity of the cytokinins by means of chemical derivatization, thereby gaining sensitivity in FAB-mass spectrometry. In paper **I**, the development of procedures to obtain hydrophobic derivatives of of the major isoprenoid cytokinins, is described. A gain in $[M+H]^+$-signal intensity was recorded following derivatization (Fig. 4a) and, in addition, the derivatives were well suited for detection in selective reaction monitoring mode (SRM), further improving the signal to noise-ratio in quantitative analysis (see 10.7).

A gain in qualitative information of the mass spectrum of derivatized cytokinins was also found, due to the formation of charged sugar fragments. In, for example, the ESI mass spectra of underivatized ZR (Fig. 3b), all fragment ions contained the purine ring, a prerequisite for charge retention. However, the derivatization increased the proton affinity of the sugar moiety and the propionyl-ZR spectrum contained the fragments m/z 301, 153 and 97, specific for a propionylated ribose moiety (Fig 4b). The gain in qualitative information is essential when structures of unknown cytokinin conjugates are to be solved. The suggested identity of an unknown iP- and Z-conjugate as a monoacetylated glucoside was, for example, based on the fragmentation pattern of the sugar moiety (Fig. 7b).

Propionic acid esters are rare in nature since naturally occurring acids are formed by the sequential addition of two-carbon units derived from acetyl-CoA. It is thus safe to perform propionylation of plant samples since the risk of misinterpreting a cytokinin propionylated *in vivo* is very low.

10.6 Capillary liquid chromatography of derivatized cytokinins

The derivatization improves the chromatographic properties of cytokinins in reversed phase chromatography on silica based stationary phases. The hydrophobic derivatives have longer retention times (Fig 4a) and give rise to

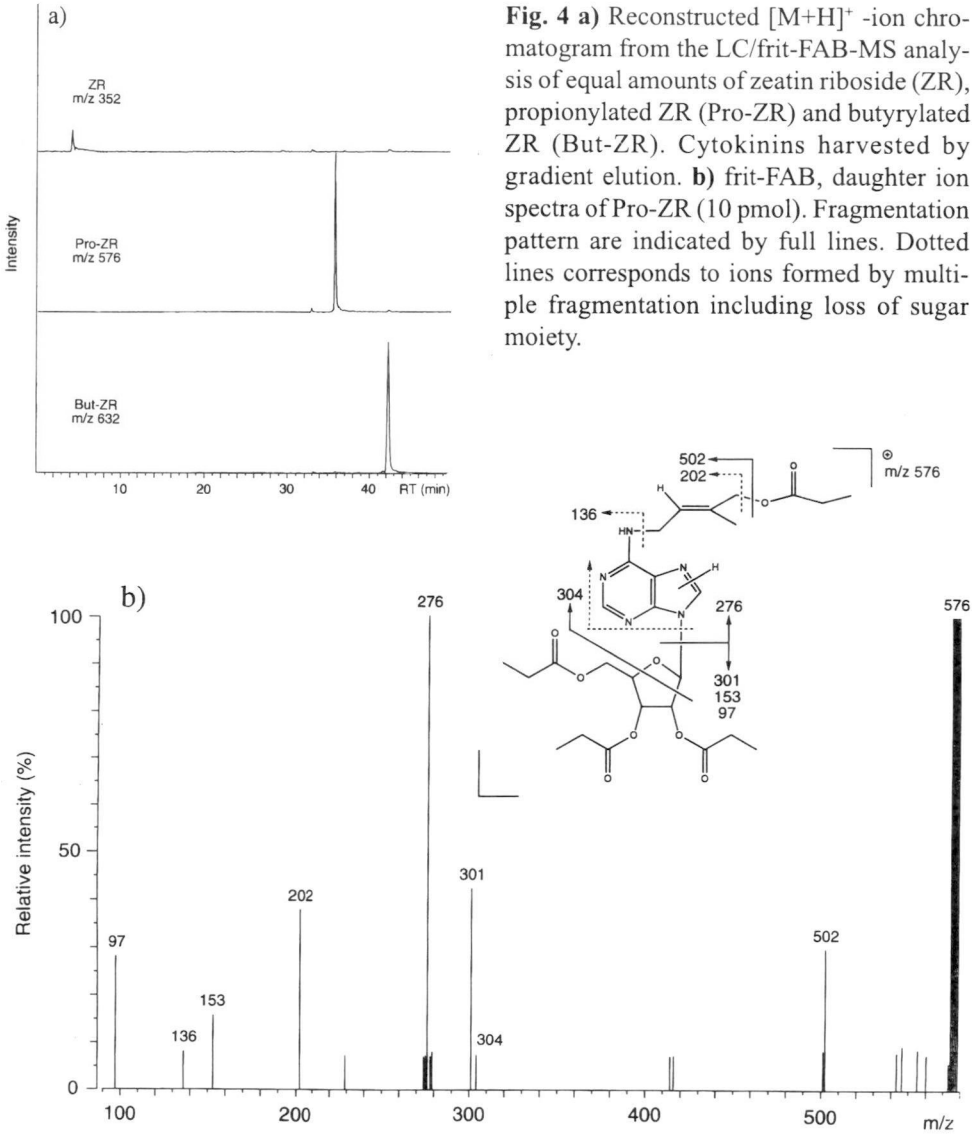

Fig. 4 a) Reconstructed $[M+H]^+$ -ion chromatogram from the LC/frit-FAB-MS analysis of equal amounts of zeatin riboside (ZR), propionylated ZR (Pro-ZR) and butyrylated ZR (But-ZR). Cytokinins harvested by gradient elution. **b)** frit-FAB, daughter ion spectra of Pro-ZR (10 pmol). Fragmentation pattern are indicated by full lines. Dotted lines corresponds to ions formed by multiple fragmentation including loss of sugar moiety.

peaks with greater symmetry. This is explained by the derivatization of amine hydrogens and/or the shielding of electron rich nitrogens by bulky groups of the derivatives, either of which reduces the interaction with acidic silanol groups in the stationary phase.

The hydrophobic character of the derivatives enables large samples to be injected onto the capillary column, which can be done by a modified Waters 717 Autosampler. Derivatized samples are dissolved in 25 µl 20% aqueous acetonitrile, 20 µl of which is injected, and this injection volume (2 x column dead volume) gives an injection efficiency of 80%. In our experiments, an autosampler, built for 0.5-2 ml min^{-1} flow rates, was integrated with a pneumatic switching valve, to enable it to function in a capillary-LC system. The internal tubing connecting valve 1 (V1) and seal

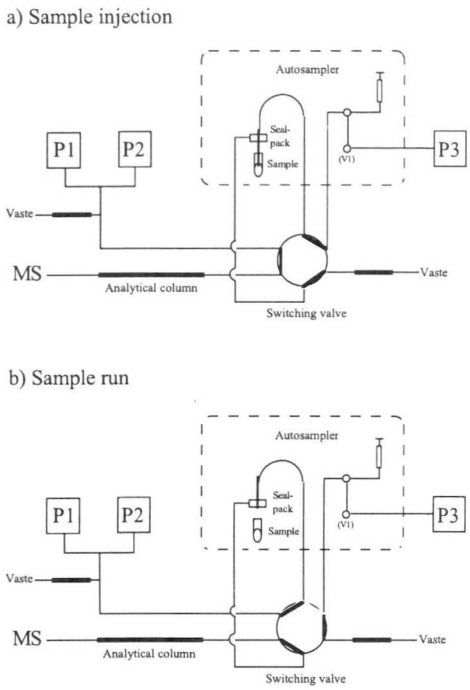

Fig. 5 Functional description of capillary liquid chromatography / autosampler setup. **a)** Injection mode: autosampler offline and preequilibrated by pump 3 (P3). **b)** Run mode: sample loop and seal pack on-line with chromatography system. (capillary LC pumps: P1+P2, mass spectrometer: MS).

pack was removed from the autosampler and the connections were plugged. The autosampler inlet was connected to a constant pressure pump setup (P3) while the sample loop and the autosampler outlet were connected to the switching valve (Fig. 5). The other connections of the valve were coupled to capillary LC pumps (P1 and P2), an analytical LC column (300 µm ID) and a vaste outlet.

The operation of this system involves two phases. The first is the injection phase, when the autosampler is offline and pre-equilibrated with 20 % aqueous acetonitrile / 1% formic acid, supplied by the third pump (Fig. 5a). Immediately after injection, the valve is reverted, thereby switching the sample loop and the seal pack online with the capillary LC column (Fig. 5b). Sample loading is performed at a flow rate of 20 µl min^{-1} for 5 min. At the end of the sample loading period, when the isocratic flow of 50% aqueous acetonitrile / 1% formic acid, pumped by the capillary LC pumps (P1+P2) starts to elute the derivatized cytokinins, the flow rate is decreased to 4.5 µl min^{-1} for the rest of the ca 30-40 min analysis time. After 10-15 min, the switching valve is returned to the "Injection" position. Following this, the liquid flow pumped by the capillary LC pumps (P1+P2), are delivered directly to the capillary column and, in parallel, the autosampler is equilibrated for the next injection by the third pump (P3).

At the injection event, the column is equilibrated with the strong mobile phase. A portion of weak mobile phase, promoting enrichment of the derivatized cytokinins, however, precedes the injected sample due to the autosampler set-up. This implies that no equilibration time is needed between samples, and each sample requires 30-40 min total analysis time.

10.7 Meta-stable ion analysis in FAB mass spectrometry

Among the gas-phase molecular ions produced by FAB-ionization, a wide spectrum of internal energies are found. In the upper energy range, molecular ions have a very short lifetime and fragments, formed in the ion source, are monitored by the mass analyser. In the low energy range, the lifetimes are much longer, and molecular ion species are detected. A fraction of molecular ions will have an intermediate internal energy and are so

called meta-stable ions. These molecular ion species are accelerated intact from the ion source, but they spontaneously decompose during the flight towards the detector.

A magnetic sector mass spectrometer operating in a B/E-linked mode will detect the meta-stable daughter ions if it is tuned for the correct parent ion. B/E-linked scanning (Kassel *et al.* 1991) and selective reaction monitoring (SRM) (Gaskell and Millington 1978) provide much higher selectivity compared to analysis of normal, ion-source derived ions. The parent ion resolution is low (R<100), due to the translational energy spread of the parent ions, but combined with daughter ion resolution of 1000, a high overall selectivity is achieved. Moreover, the selectivity increases when the relative size of the daughter ions decreases (Chapman 1993). However, this improvement in resolution is balanced by a reduction in detector response due to the lower kinetic energy of the smaller daughter ions. The optimal signal/noise ratios in SRM are often found for daughter ions that have around 50% of the parent ion mass, due to the trade-off between selectivity and signal intensity. The improved performance of the cytokinin derivatives in SRM analysis is thus due to the combined effects of improved parent-ion formation, increased parent-ion size and diagnostic transitions well suited to the SRM measurement mode.

10.8 Isolation of cytokinins from plant material

Plant materials differ in their contents of pigments, phenolic compounds and other secondary metabolites that will have an impact on the recovery and purity of the

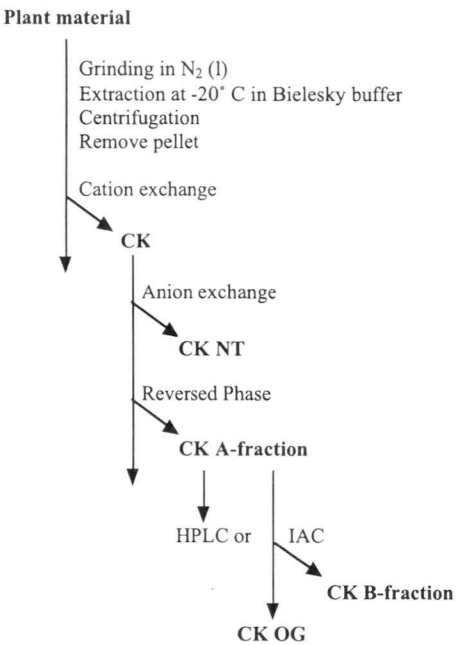

Fig. 6 Schematic protocol for cytokinin isolation from plant tissue. Split arrows describes binding to cation or anion exchange-, reversed phase- or immunoaffinity-columns (IAC). (CK: all adenine cytokinins, CK NT: cytokinin nucleotides, CK A: Non-nucleotide cytokinins, CK B: Cytokinin bases, nucleosides and 9-glucosides, CK OG: cytokinin-O and 7-glucosides).

cytokinins after isolation. A method developed for one plant material is thus not necessarily suitable for another plant material. Several different purification procedures have been used in the studies described here, but the most recent is considered the most useful. This method was developed and optimised for *Arabidopsis thaliana* plant material (Fig. 6) (**I**) but it was also successfully applied to *Chenopodium rubrum* cell suspension cultures (11.2).

The extraction is performed in Bielesky buffer at –20° C to prevent degradation of cytokinin nucleotides by ubiquitous

phosphatases (Bielesky 1964). This buffer, containing 60% methanol, 25% chloroform, 10% formic acid and 5% water, has a very high extraction capacity, but it has become less frequently used in recent years, presumably due to problems associated with the subsequent cytokinin isolation (Horgan and Scott 1987). The high content of lipophilic material in the extract makes evaporation of the solvent problematic and hydrophobic cytokinins are often lost with the bulk. To circumvent these problems, we developed a purification procedure in which the protonated cytokinins in the Bielesky extract are trapped on a cation-exchange column of SCX-type (**I**). The benzenesulfonate-anion groups of the column strongly interact with the protonated purine moiety of the cytokinins, presumably by mixed ion-ion and π-electron interactions, and the cytokinins are retained, while lipophilic substances are washed away with the buffer. This is an extraordinary step in terms of purification efficiency, recovery (>90%) and ease of handling. The cytokinins are further separated by anion exchange (in which cytokinin nucleotides (CK NT), are retained), hydrophobic interaction (retaining cytokinin bases, ribosides and glucosides (CK A)) and high performance liquid chromatography (HPLC) (Fig. 6) (**I**). For a review of these methods see Horgan and Scott (1987). As a further improvement, the HPLC-separation of the CK A-fraction was replaced with immunoaffinity-chromatography (IAC) generating two fractions: a retained fraction containing bases, ribosides and 9-glucosides (CK B) and a non-retained fraction containing O- and 7-glucosides (CK OG) (**II**, **III** and **IV**) (Goicoechea *et al.* 1995).

11. Qualitative Cytokinin Analysis

11.1 Isoprenoid cytokinins in *Arabidopsis thaliana*

Ten isoprenoid cytokinins were identified by normal scan- and B/E-linked scanning-techniques in extracts of *Arabidopsis* plants. Among the cytokinins were zeatin- and isopentenyl- type nucleotides, nucleosides, free bases and glucosides (**I** and Fig. 7a). The properties of the new derivatives (see 10.5) improved the identifications, and a novel type of cytokinin, a conjugate of iP and Z, was also detected. The interpretation of the mass spectra of the iP-conjugate (Fig. 7b) suggests it is a monoacetylated glucopyranoside conjugate. The position of the acetyl group is not determined and could be 2', 3' or 4', based on the fragmentation pattern (Guevremont and Wright 1988). Dihydrozeatin species were not detected in wildtype plant material, but analysis of *ipt*-transgenic *Arabidopsis* plant material indicated they were formed at a low rate during cytokinin overproduction (**II**).

11.2 Aromatic cytokinins in *Chenopodium rubrum*

Propionyl derivatives of aromatic cytokinin nucleosides and glucosides were obtained using the procedure developed for isoprenoid cytokinins (**I**). When the derivatized aromatic cytokinins were analysed by LC/frit-FAB-MS, around 20-fold higher sensitivities than for the underivatized cytokinins were recorded. The mass spectra of the derivatized aromatic cytokinins were also enriched by additional sugar fragment ions, thereby improving the method as a tool for qualitative analysis.

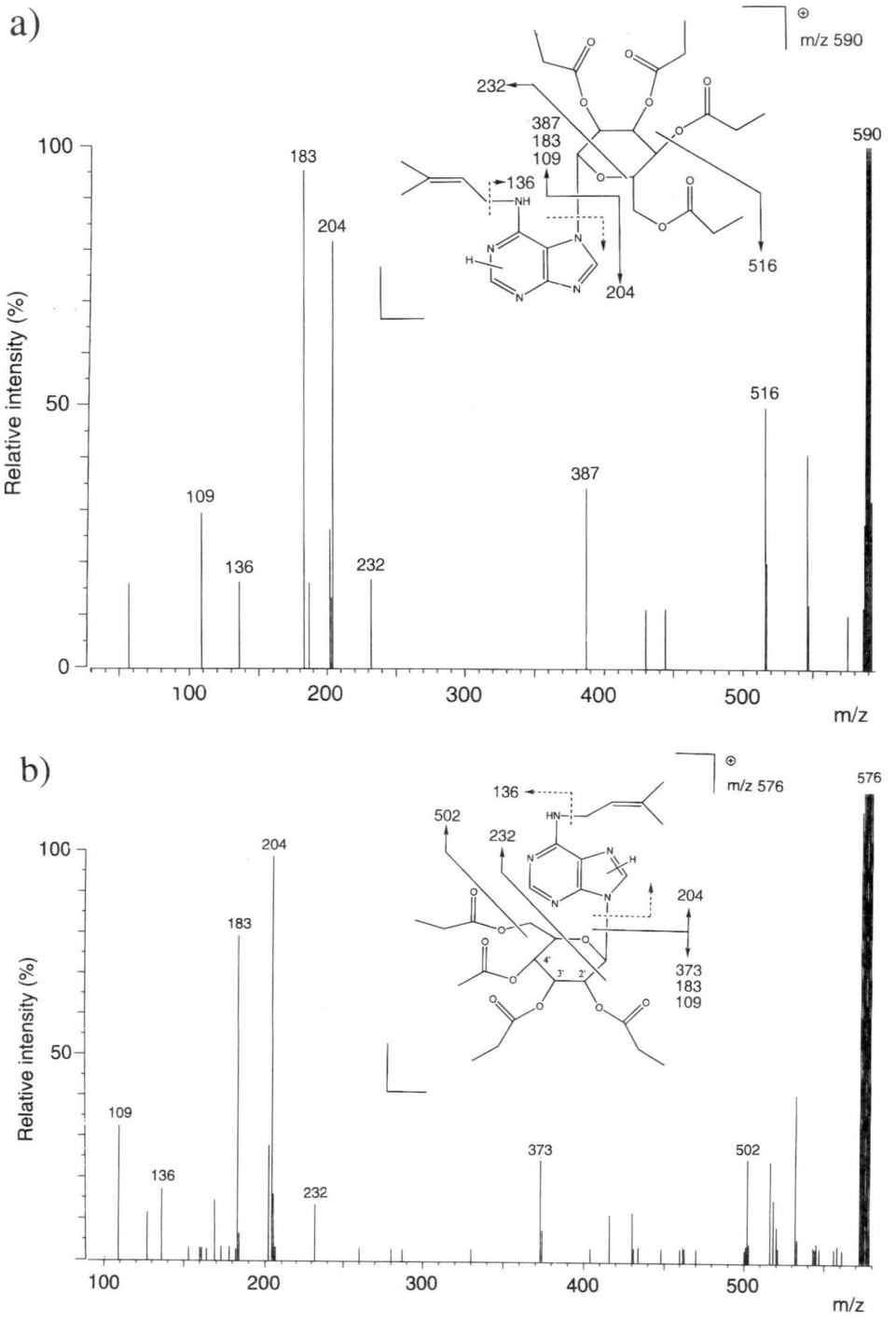

Fig. 7 Daughter ion mass spectra of isoprenoid cytokinins purified from *Arabidopsis thaliana* plant material. Samples propionylated prior analysis by LC/frit-FAB-MS **a)** isopentenyladenine-7-glucoside (iP7G). **b)** monoacetyl-iPNG (structure not verified by analysis of synthetic standard).

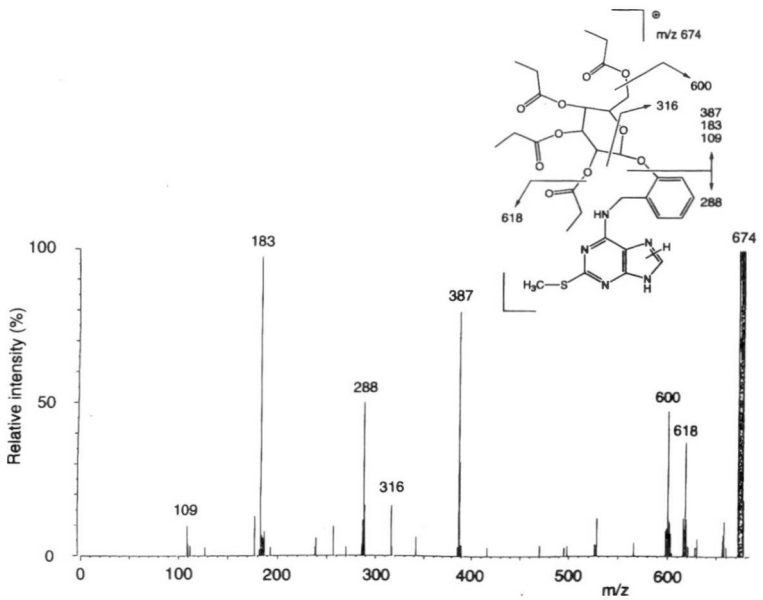

Fig. 8 Dauhter ion spectrum of 6-[2-(β-D-glucopyranosyloxy) benzylamino]-2-methylthiopurine purified from *Chenopodium rubrum* cell suspension culture and propionylated prior analysis by LC/frit-FAB-MS. Structure verified by analysis of synthetic standard.

The technique was used to identify aromatic cytokinins in photoautotrophic cell-suspension cultures of *Chenopodium rubrum*. The cell culture has well characterised growth phases (Beck and Renner 1990) and the identifications were partial achievements in the work of Karel Dolezal, aiming to clarify the involvement of aromatic cytokinins in the regulation of cell division in *Chenopodium* cell culture. Three previously undetected metabolites of aromatic cytokinins were identified; 6-[2-(β-D-glucopyranosyloxy) benzylamino]-2-methylthiopurine (Fig. 8), 6-[2-(β-D-glucopyranosyloxy) benzylamino]-purine, and 6-benzylamino-9-β-D-glucopyranosylpurine (BAP9G)(data not shown for the latter compounds). The identity of the compounds was verified by organic synthesis, performed by Jan Hanus, (Isotope Laboratory, Inst. of Exp. Botany, Prague, Czech Republic).

The identity of the aromatic cytokinins as endogenous compounds in plants, has sometimes been questioned (Strnad 1997). Our future aim is to perform *in-vivo* deuterium labelling experiments (**II**) with *Chenopodium* cell cultures. Preliminary data indicates that deuterium atoms are incorporated into aromatic cytokinins and a larger set of data could provide evidence on their existence as endogenous compounds.

12. Quantitative Cytokinin Analysis

12.1 Cytokinin quantification in *Arabidopsis thaliana*

The new FAB-method enabled quantification of nine isoprenoid cytokinins in shoot tissue of individual *Arabidopsis* plants, grown in short-day conditions for seven weeks (**I**). This report was the first quantification of cytokinins in wildtype

Arabidopsis plant material by mass spectrometry. To quantify the cytokinin bases, 250 mg plant tissue was needed while the cytokinin nucleotides, nucleosides and glucosides could be quantified in less than 50 mg of plant tissue. No breakdown of nucleotides during sample purification was detected and nucleotide/nucleoside–ratios around 14 were found for both iPAMP/iPA and ZR/ZRMP.

The endogenous cytokinin levels found in *Arabidopsis* were generally in agreement with earlier reports, levels of around 1-2 pmol g^{-1} for the cytokinin bases and nucleosides being found. The nuceotides and glucosides were present at higher levels (10-70 pmol g^{-1}) and, as a general trend, the zeatin-type of cytokinins were present at 2-fold higher levels than their isopentenyl analogues.

The quantification method was also used to characterize transgenic *Arabidopsis* plants expressing the *ipt*-gene under the control of a glucocorticoid inducible expression system (Aoyama and Chua 1997). Three transgenic plant lines (designated 5-1, 2-7, and 3-2), constructed by Qun Wang, were investigated. The plants showed cytokinin-overproducing phenotypes ranging from weak to strong when grown in inductive conditions. Cytokinin quantification of induced plants showed that the levels of Z-type cytokinins correlated with the phenotypes observed (Fig. 9). Based on the quantification data, plant lines were chosen for further experiments (**III**) and (15.6).

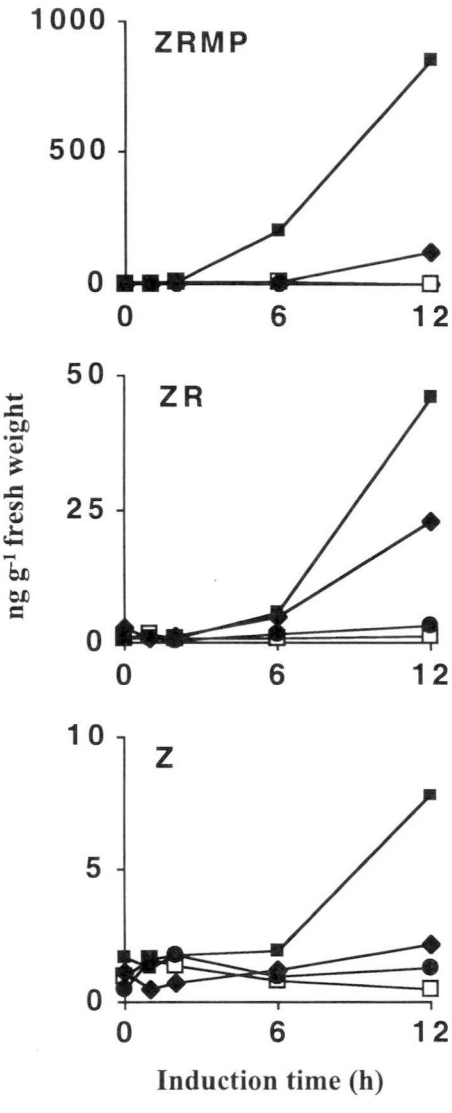

Fig. 9 Response of cytokinin levels to induced ipt-expression in transgenic *Arabidopsis* plants expressing the *ipt*-gene under the control of a glucocorticoid inducible expression system. Three transgenic plant lines; 3-2 (closed squares), 2-7 (closed diamonds) and 5-1 (closed circles) and wildtype were investigated in the study.

12.2 Cytokinin quantification in *Nicotiana tabacum*

Cytokinin analysis of wildtype and transgenic tobacco was performed by a further development of the LC/frit-FAB-MS-method described by Imbault *et al.* (1993). It included precolumn acetylation of cytokinins and was essentially described in Moritz (1995). The method generated data on the pooled content of cytokinin nucleotides and nucleosides, and only the iP- and Z- types of cytokinins were quantified (**V**, **VI**, **VIII**).

13. Tracing *De-Novo* Cytokinin Biosynthesis

13.1 Development of a new tool for analysis of cytokinin biosynthesis

To enable new experiments to be performed on the biosynthesis of cytokinins, a technique for *in-vivo* deuterium labelling of cytokinins was developed (**II**). *Arabidopsis* plants grown in aseptical liquid media were replaced on media enriched with 30% deuterium oxide (^2H_2O) and deuterium incorporation into cytokinins was analysed by mass spectrometry. The plants rapidly incorporated deuterium atoms into the sidechain and the sugar moieties of cytokinin nucleotides. The adenine portion of the cytokinins was essentially unlabelled after the 3-24 hr incubation periods, presumably due to recycling of the adenyl compound in the plant cell via the adenine salvage pathway (Hirose and Ashihara 1984; Lee and Moffatt 1994). Mass isotopomer analysis of the cytokinin base fragments thus essentially detects label in the cytokinin sidechain, the structural theme that also confers cytokinin activity. A mass isotopomer analysis method, based on SRM, was developed for the zeatin-class of cytokinins (**II**). The high selectivity and sensitivity of the method (see also 10.6) enabled analysis of cytokinin biosynthesis in wildtype *Arabidopsis* plants (13.3, 14.3). Deuterium oxide has, as discussed in section 9.2, very good properties as a tracer, and the biosynthesis monitored is reliable due to the selectivity provided by the mass spectrometer. Since the rate of tracer incorporation is related to the turnover of the endogenous pools, this technique allowed, for the first time, measurement of cytokinin biosynthesis rates.

13.2 Measurement of biosynthesis rates

The biosynthesis rate of ZRMP was calculated in an *ipt*-expressing *Arabidopsis* plant line, using a fractional synthetic rate approach (Toffolo *et al.* 1993) (**II**). ZRMP was chosen since it is a major cytokinin, produced early in the biosynthesis pathway, and it is believed to be formed by specific hydroxylation of iPAMP (Fig. 2) (McGaw and Burch 1995; Chen 1997). This conversion is believed to be unidirectional and the precursor pool (iPAMP) is accessible for measurement, two factors facilitating calculation of biosynthesis rates. From the mass isotopomer data, the change in deuterium enrichment in ZRMP ($z_B(t_2)$-$z_B(t_1)$) was calculated in the time span 3-6 h and the change of the deuterium enrichment in iPAMP was described as a function of time, $z_A(t)$. Using equation 1, a biosynthetic rate of 18 ng g^{-1} h^{-1} was calculated for ZRMP, and a 25 h turnover of the ZRMP pool (**II**).

By using an alternative method, in which theoretical, full precursor enrichments are assumed (Bialek *et al.* 1992), the biosynthetic rate of iPAMP and ZRMP was estimated to 6 and 9 ng g^{-1} h^{-1}, respectively. This method normally gives rise to underestimated rates of synthesis but is however useful when the precursor is not accessible for mass isotopomer analysis.

Eq 1. $$FSR = z_B(t_2) - z_B(t_1) / \int_{t_1}^{t_2} z_A(t)dt$$

13.3 Cytokinin biosynthesis in *Arabidopsis* roots and shoots

The new method for mass isotopomer analysis by SRM (**II**) was used to investigate biosynthesis of ZRMP in wildtype *Arabidopsis thaliana* plants, ecotype Colombia. Plants were first grown in sterile conditions on soft agar and, prior to ^2H_2O -incubation, they were harvested and divided into root- and shoot-portions. Pooled root- and shoot- tissues (5g) were incubated separately to prevent translocation of *de-novo* synthesised cytokinin species.

Strong *in-vivo* deuterium labelling of ZRMP was detected in the root, and thus *de-novo* cytokinin biosynthesis was unequivocally detected for the first time in a wildtype plant (Fig. 10b) (Letham 1994). Strong *in-vivo* deuterium labelling of ZRMP was surprisingly also found in the shoot sample, suggesting that cells in the aerial plant parts also have the capacity for cytokinin synthesis (Fig. 10a). It has been suggested earlier that all meristematic tissues have this capacity, but evidence has been lacking (Chen *et al.* 1985).

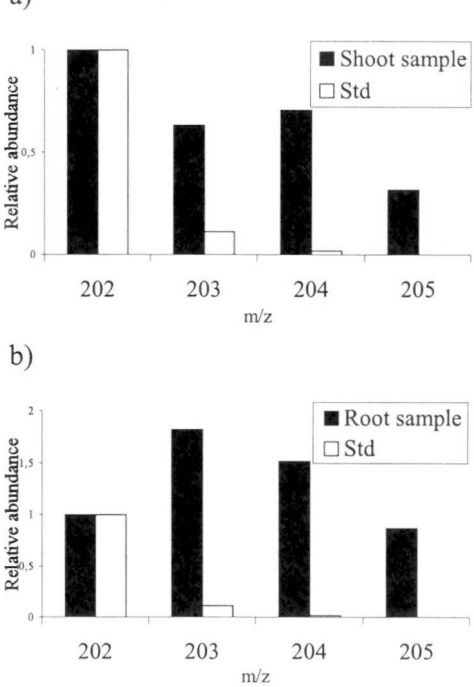

Fig. 10 *In-vivo* deuterium labelling of cytokinins in wildtype *Arabidopsis* plants. Cytokinin de-novo biosynthesis detected in a) shoot tissues and b) root tissues after 48 h incubation of detached plant parts in growth media enriched with 30% ^2H_2O.

The ZRMP turnover is apparently slightly higher in the root tissue, but more data is required to confirm this calculation. The situation is also complicated by the fact that IAA, transported from the apical shoot, probably negatively regulates the cytokinin biosynthesis in the root, as suggested by Bangerth (1994). Excised roots will thereby have much higher cytokinin biosynthesis rates than roots of the intact plant.

14. An Alternative Cytokinin Biosynthesis Pathway

14.1 Isopentenyltransferase-mediated cytokinin biosynthesis

The new method of *in-vivo* deuterium labelling was used to investigate cytokinin biosynthesis in an *Arabidopsis* line expressing the *ipt*-gene under the control of its natural promoter (13.2)(**II**). However, the labelling data did not fully confirm the established model for cytokinin biosynthesis. There are at least three different metabolic fates of iPAMP in plant cell (Fig. 2). The conversions are; hydroxylation to produce ZRMP, dephosphoraylation to form iPA and oxidative degradation of the sidechain, with AMP as the product. This implies that the biosynthetic rate of iPAMP has to be higher than the rate of ZRMP-formation, to maintain the iPAMP-level in the plant cells. The competetive metabolic activity is expected to be high in the *ipt*-line since increases cytokinin oxidase activity has been linked to cytokinin-overproduction (Motyka and Kaminek 1994; Zhang *et al.* 1995).

However, the rate of iPAMP-biosynthesis (6 ng g^{-1} h^{-1}) was not even large enough to explain the rate of ZRMP-formation (9 ng g^{-1} h^{-1}) when other metabolic iPAMP-conversions are neglected. It was thus clear that high iPAMP-turnover rates can not explain the ZRMP-accumulation.

After this work was performed, the existence of an alternative, iPAMP-independent pathway of ZRMP biosynthesis was identified (**III**) and the parallel biosynthetic flux explains the quantitative data above (13.2)(**II**).

14.2 iPAMP-independent cytokinin biosynthesis

Using the *in-vivo* deuterium labelling method for analysis of cytokinin biosynthesis (**II**) and a transgenic plant line with inducible *ipt*-expression, we were able to obtain evidence of the existence of an alternative, iPAMP-independent ZRMP-biosynthesis pathway (**III**). The *in-vivo* labelling was essential to enable labelling in the alternative pathway, since the associated sidechain precursor has not yet been identified. When plants with induced *ipt*-expression were grown on media containing 30% ^2H_2O, higher deuterium enrichment was observed in ZRMP than in iPAMP. This labelling pattern is impossible if iPAMP is the only precursor of ZRMP, and implies that ZRMP has other precursor(s) rather than cytoplasmic iPAMP. Further, labelling with ^2H_2O + [$^2H_6$]iPA allowed independent labelling of the iPAMP and ZRMP pools, thereby corroborating the absence of rapid iPAMP-ZRMP conversion, and showing that the major portion of the ZRMP-pool was synthesised by an iPAMP-independent route (**III**).

The reason for using the inducible *ipt*-expression system in these experiments was to avoid the high cytokinin-oxidase activity found in constitutive cytokinin-overproducing plants (Motyka and Kaminek 1990). If the mechanisms preventing iPAMP-accumulation are induced by cytokinin overproduction, at least a transient increase in iPAMP-content would have been expected before the homeostatic mechanisms were initiated. No such iPAMP-accumulation or pulse was observed and the precursor-product relationship between iPAMP and ZRMP was therefore evaluated.

From our study, it is evident that the major reaction catalysed by the bacterial isopentenyltransferase not is the transfer of an isopentenyl sidechain. Induction of *ipt*-expression did not significantly alter the rate of deuterium incorporation in iPAMP, in contrast to the incorporation into ZRMP. It is also possible that the detected *de-novo* iPAMP-production detected was only formed *via* endogenous biosynthesis pathway(s). The absence of increased iPAMP levels and altered iPAMP-deuterium incorporation, following induction of *ipt*-expression, support this hypothesis.

The identity and the biosynthetic origin of the sidechain precursor associated with the alternative pathway remain unknown. 4-pyrophosphate-2-methyl-*trans*-but-2-enol is a possible candidate, and maybe formed as a branch-off from the terpenoid pathway. However, no label was detected in cytokinins of grown-gall tissue when fed with labelled mevalonic acid, a precursor in the terpenoid pathway (Palni *et al.* 1983). The result could be explained by the complexity of the terpenoid pathway and the presence of other terpenoid precursors with potentially larger importance than mevalonic acid.

14.3 Two biosynthesis pathways in wildtype *Arabidopsis*

Isotopomer analysis by SRM (**II**) also enabled an investigation of the biosynthesis pathways in wildtype *Arabidopsis* plants. From ^2H_2O + [$^2H_6$]iPA double-tracer experiments it was evident that an alternative, iPAMP-independent pathway of ZRMP-biosynthesis also exists in wildtype *Arabidopsis* plants (**III**). The activity of the alternative pathway was much lower than in *ipt*-expressing plants, and of about equal importance to the iPAMP-dependent pathway. The experimental design could, however, give rise to an overestimation of the impact of the iPAMP-dependent pathway. In the experiment, the total iPAMP-pool increased due to the tracer used, [$^2H_6$]iPAMP, and the larger pool-size probably increased the iPAMP-ZRMP conversion rate. Thus, the alternative, iPAMP-independent pathway may have a relatively greater importance than indicated by these data. The relative contribution of the biosynthesis pathways may also vary in a tissue- and time- dependent manner and it will be the subject of future investigations.

Feedback regulation of a rate-limiting step provides efficient regulation of a biosynthesis pathway. The possibility that feedback regulation of the alternative pathway by ZRMP occurs is a hypothesis that is partially supported by the data gathered. In the ^2H_2O + [$^2H_6$]iPA double-tracer experiment, a significantly higher level of *in-vivo* deuterium labelling of ZRMP was detected when an inhibitor that blocks iPAMP-ZRMP conversion was added. Inhibition of the iPAMP-dependent pathway prevents [$^2H_5$]ZRMP-formation, and reduces the total ZRMP pool size. Under these conditions, up-regulation of the *de-novo* ZRMP-biosynthesis was detected, in accordance with the feedback-regulation model. An experiment in which the ZRMP-pool size is manipulated by a tracer, at the same time as the *in-vivo* deuterium incorporation is measured, would provide more information about this putative regulation.

15. Tobacco With Moderate Cytokinin Overproduction

15.1 Generation of *ipt*-transgenic plants

My first project as a graduate student involved the characterisation of tobacco lines expressing the *Agrobacterium ipt*-gene. The lines were constructed by Staffan Eklöf (1996) through transformation of *N. tabacum* L. cv. W38 with a promoter-trap, *ipt*-vector. This as an approach to generate plants that weakly expressed the *ipt*-gene (**IV**). At that time, the *ipt*-gene in most *ipt*-transgenic plants was under the control of strong, constitutive promoters. These plants showed severe phenotypic alterations, including absence of root formation, preventing regeneration of intact plants (Smigocki and Owens 1988; Smigocki and Owens 1989; Beinsberger *et al.* 1991).

The aim was to generate *ipt*-expressing plants with a mild phenotypic deviation from wild-type for studies of hormone metabolism in plants with steady-state rates of cytokinin production close to the limit of the plant's homeostatic control system. Two plant lines, P3 and P9, were selected based on their moderately altered phenotypes, which made it possible to regenerate intact, fertile plants. Both lines had a reduced level of root formation, but were still able to grow on soil in the greenhouse. When the general expression pattern of the P3-promoter was studied, expression was mainly localised to young leaves and the apical stem region, although even in these tissues the level of expression was weak (**IV**). The promoter has not been identified and the cell-specific expression in these plants remains to be fully characterised.

15.2 Analysis of cytokinin overproduction

We detected a 10-fold increase of the ZR+ZRMP levels in young tissues of P3, this in accordance with northern hybridisation data, supporting the observation that activity of the P3-promoter is most pronounced in young tissues (**IV**). The Z level in young leaves of P3 was very close to wild-type levels (1.5-2-fold) in contrast to Z7G, the inactive conjugation product, which accumulated 6-10-fold in young leaves of P3. The moderate alteration of Z, the putative active form (Brinegar 1994) indicated that the cytokinin overproduction in P3 is whithin the limits of the homeostatic system. The Z7G-levels remained high in old leaves, which were depleted in active cytokinin forms, supporting the view of Z7G as a product of irreversible inactivation (Letham *et al.* 1983).

15.3 Analysis of cytokinin metabolism

The cytokinin profile in young leaves from P3 plants (Fig 5b in **IV**) indicated that Z-turnover is up-regulated in the P3-line. Thus, the steady-state Z-levels are not dramatically different from wildtype, despite the fact that the precursors, ZRMP + ZR, are present at levels 10-fold higher than in wildtype plants. The data indicated that Z-metabolism is upregulated to maintain close-to-wild-type levels of Z, even with an increased rate of cytokinin biosynthesis.

To test this hypothesis, a cytokinin-metabolism study was performed after feeding leaf discs of young leaves from wildtype and P3 with [^{3}H]Z, essentially according to the method used in publication **V**.

The growth conditions were modified since the *ipt*-plants grow slowly in *in vitro*-conditions (Li et al. 1992, Eklöf et al. 1996). However, the growth rate was partially restored when P3 plants were grown in *in-vitro* conditions permitting transpiration. To enable this, sterile filters were mounted in the modified screw-cap aperture of 1200 cm^3, polymeric Erlenmeyer flasks. A 6.5 cm ∅ hole was opened in the bottom of the flasks to allow gas exchange *via* a cotton bung. Autoclaved flasks were placed upside down in large beakers and were filled with 200 cm^3 of autoclaved, Murashige and Skoog medium/1% agar (Murashige and Skoog 1962). To compensate for moisture lost via the cotton bung, the beaker was filled with water.

Wildtype and P3 leaf discs, fed with radioactive [U-^{3}H]Z for 3 h, were harvested and extracts were analysed by HPLC/RC. The resulting chromatograms showed qualitative and quantitative differences between the two lines (Fig. 11). In the P3 tissue, only minute amounts of [^{3}H]Z remained unmetabolised after 3 h of incubation, in contrast to wildtype samples, where the major part of the radioactivity coeluted with [^{3}H]Z. Thus, these data support the hypothesis that the rate of Z-metabolism was altered in P3 plant tissues.

Radioactive Z-metabolites from P3 plant material coeluted with adenine, adenosine, Z7G, Z9G and ZR. The major metabolite had a retention time approximately equal to that of ZOG, but addition of radioactive ZOG to samples prior to analysis gave rise to a ZOG peak with a slightly longer retention time (data not shown). Strong acidic hydrolysis (TFA, 100°C, 1h) modified the unknown metabolite, and the radioactive product coeluted with Z after this treatment (data not shown). No attempt to identify the metabolites by mass spectrometry was however been performed.

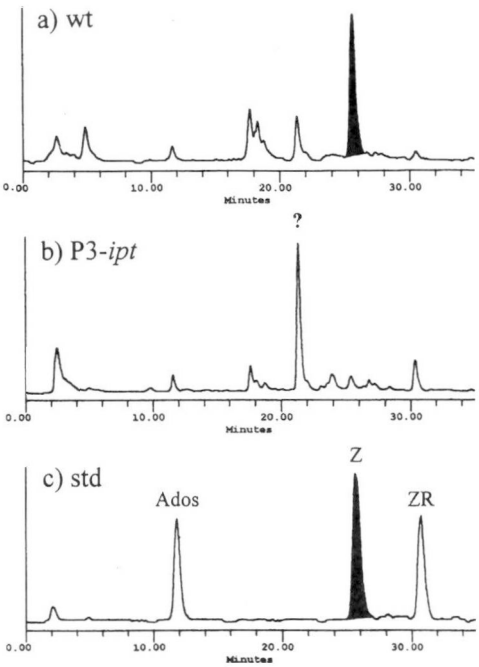

Fig. 11 Metabolism of [^{3}H]Z in leaf discs from **a)** wildtype-and **b)** P3-plants, incubated in darkness for 3 hrs. Cytokinin extracted and analysed by HPLC / RC.

The formation of [^{3}H]adenine and [^{3}H]adenosine as a minor metabolites in both wildtype and P3 plant material, was consistent with the lack of significantly increased cytokinin oxidase activity found in P3 extracts (**IV**). The data from the P3 line thus indicated that sidechain cleavage is not an important mechanism for cytokinin inactivation during vegetative growth.

15.4 IAA content and metabolism

Reduced levels of free IAA were found in young tissues of both the P3 and the P9 *ipt*-expressing lines compared to wildtype plants, and the reduction in IAA-levels (**V**) correlated with the

degree of cytokinin overproduction (**IV**). This was in agreement with findings from another transgenic tobacco line with a low level of *ipt*-expression (Yusibov *et al.* 1991). However, our study took the IAA-analysis a step further by investigating in detail the regulation of the IAA-pool size in P3-plants.

In order to elucidate any qualitative shift in the IAA metabolism, radioactive 2'-[^{14}C]IAA was fed to leaf discs of wildtype and P3 plants and the discs were incubated for 12 or 24 hrs. The samples were analysed by HPLC-RC, and the metabolic profiles were found to be essentially identical in the two sets of plant material (**V**).

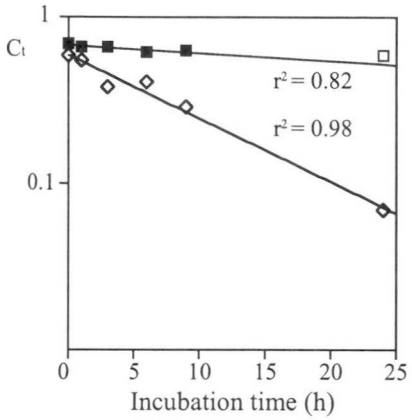

Fig. 12 Decay of [$^{13}C_6$]IAA in P3(closed squares) and wildtype (open diamonds). Semilogarithmic plot of Ct ([$^{13}C_6$]IAA/[$^{12}C_6$+$^{13}C_6$]IAA) vs incubation time. Excluded P3-data point indicated as an open square.

15.5 IAA turnover and biosynthesis

As the next step, the IAA turnover in cytokinin-overproducing (P3) and wildtype plants was investigated by supplying an IAA-tracer to the plant material and monitoring its rate of disappearance. When [$^{13}C_6$]IAA was fed to the apical shoot of wildtype and P3 plants and the decay in the [$^{13}C_6$]/[$^{12}C_6$]IAA ratio was monitored, a lower rate of IAA turnover was detected in the P3 than in the wildtype plants (Fig. 4 in **V**). The decay in isotope ratios has been shown to obey first order kinetics and thus a semilogarithmic plot of the [$^{13}C_6$]IAA/[$^{12}C_6$+$^{13}C_6$]IAA-ratio generates a straight line (Tam *et al.* 1995) (**VI**). When the [$^{13}C_6$]IAA-turnover data in publication **V** was reanalysed according to the methods in paper **VI**, the wild-type data were also consistent with those expected for a first-order reaction, and the semilogarithmic data had a very high regression coefficient (r^2=0.98)(Fig. 11). The decay in isotope-ratios of the full P3-data set did not, however, obey first order kinetics. To improve the linear fit of the P3 semilogarithmic data, the 24 h data point was excluded and a r^2-value of 0.82 was derived (Fig. 12). From the coefficients of the regression lines, the IAA half-lifes in wildtype and P3 were calculated ($t_{1/2} = \log 2\ k^{-1}$) to be 7.9 h and 60 h, respectively.

In a developmental phase where the endogenous level of IAA is stable, the rate of IAA-turnover will essentially be equal to the rate of IAA biosynthesis. The IAA-biosynthesis rate in P3 and wildtype plants was therefore compared by the use of ^2H_2O as a tracer. This tracer lack the drawbacks mentioned above, and has previously been used to investigate IAA-biosynthesis (Pengelly and Bandurski 1983; Bialek *et al.* 1992; Jensen and Bandurski 1996).

When plants were incubated with ^2H_2O-enriched growth media, a significantly higher rate of *in-vivo* deuterium incorporation into IAA was detected in wildtype than in P3-plants (**V**).

The deuterium enrichment of the IAA-precursor will, as discussed above (13.2), have an impact on the amount of labelled IAA produced. Deuterium enrichment in Trp was used as an indicator of the deuterium enrichment of the IAA-precursor, although tryptophan (Trp) is no longer believed to be the major precursor of IAA (Normanly *et al.* 1995). In the Trp-independent route to IAA, an indolic Trp precursor, possibly indole-3-glycerol phosphate or indole, is believed to acts as a branch point for IAA biosynthesis without prior conversion into Trp (Wright *et al.* 1991). A difference in the deuterium enrichment in Trp will, however, reflect a difference in the general indolic metabolism. The IAA-biosynthesis rates of the plant materials were estimated by reanalysing the deuterium enrichment data according to a fractional synthetic rate-approach (Toffolo *et al.* 1993) (see 13.2). To enable this calculation, linear changes of the IAA- and Trp- deuterium enrichments for the 24h incubation period and insignificant losses of label through IAA-metabolism were estimated. The enrichment data, transformed to tracer/tracee ratios (z) gave rise to fractional synthetic rates of 9.4% h^{-1} for wildtype and 5.9% h^{-1} for P3 when applied in equation 1. The deduced IAA half lives were 5.3 h for wildtype and 8.4 h for P3 plants, and the corresponding biosynthesis rates were approximately 3.6 ng g^{-1} h^{-1} and 1.2 ng g^{-1} h^{-1}, respectively.

Thus, we have shown, by two separate methods, that the rate of IAA-turnover is lower in the cytokinin-overproducing P3 plants than in wildtype. The estimated half-life differs, depending on the method used for calculation and the variation is partially explained by the assumptions applied to enable the calculation and the method error linked to the experimental systems.

The lower biosynthesis rate largely explains the lower levels of free IAA found in the P3-tobacco line. However it could not be excluded that metabolic activity may have been maintained at the wildtype level in young apical shoots of P3-plants, since higher levels of conjugated-IAA were detected in this part of the plants.

15.6 Effects on IAA homeostasis by induced cytokinin overproduction

The downregulation of IAA-biosynthesis found in the constitutive P3 cytokinin-overproducer could be mediated *via* a direct effect of elevated cytokinin levels on expression levels of endogenous IAA-biosynthesis genes or the activity of the encoded enzymes. Alternatively, a more general shift in development could have been induced, resulting in a lower growth rate and thus, a lower IAA-biosynthesis rate. To investigate these possibilities, cytokinin overproduction was induced in three transgenic *Arabidopsis* lines with inducible *ipt*-expression (12.1) and their IAA levels were analysed in a time study. The aim was to investigate the directness of the effect of cytokinin-overproduction on IAA-levels. A lag of 48 hrs was recorded between induction of *ipt*-expression and the time when significantly reduced IAA-levels was detected. This indicated that the cytokinins had an indirect effect, probably mediated *via* alterations in growth and development (data not shown).

16. Tobacco with Strong Auxin-Overproduction

16.1 Auxin-overproducing tobacco

A number of transgenic tobacco plants, expressing the *Agrobacterium tumefaciens iaaM* and *iaaH*-genes were constructed by Folke Sitbon and used as models for studies of IAA metabolism (Sitbon 1992). In the following experiments, two tobacco transformants, 35S:*iaaM* x 35S:*iaaH,* derived from *N. tabacum* L. cv. W38 or L. cv. Petit Havana SR1 plants, were used. Both lines were crosses of transformants of *N. tabacum*, that separately carried the *A. tumefaciens iaaM* and *iaaH*-genes driven by the 35S CaMV promoter. The SR 1-line is described in Sitbon *et al.* (1993) and the W38-line was briefly described in Sitbon (1992). Both of these plant lines express a traits typical of auxin-overproducers including: adventitious root formation, strong apical dominance, epinastic, stiff leaves with erect blades and thin, slightly shorter internodes than wildtype plants (Sitbon *et al.* 1992).

16.2 Cytokinin content and metabolism

To study the effect of auxin-overproduction on endogenous cytokinin levels we investigated the IAA-overproducing W38 tobacco line described above (16.1).

A significant reduction in the steady-state cytokinin contents was found in the IAA-overproducing tobacco line and the reduction in ZR+ZRMP-content was pronounced in both leaves and stem (**V**). Earlier data have shown that application of auxins to plant tissue can markedly reduce their cytokinin content, and a regulatory mechanism including IAA-induction of cytokinin oxidase activity was suggested to mediate the response (Palni *et al.* 1988; Motyka and Kaminek 1990; Zhang *et al.* 1995; Redig *et al.* 1997). However, contrary to this hypothesis, a lower level of cytokinin oxidase activity was detected in IAA-overproducing leaves than in wildtype leaves. In contrast to many of the previous studies, we have assayed cytokinin oxidase purified from intact plants grown in the green-house under close to natural conditions. Very different cytokinin oxidase activity may be expressed under tissue culture conditions, and results obtained under such conditions may not be relevant to the *in planta* situation.

To investigate cytokinin metabolism in the auxin-overproducing plants, leaf discs taken from them, and from wildtype plants, were fed with [U-^{3}H]Z and incubated for 6 or 12 hrs. The samples were analysed by HPLC/RC to monitor cytokinin conjugation and oxidative-sidechain cleavage, and the metabolic profiles were essentially identical. Thus, no qualitative difference was detected between the two sets of plants, and there were no indications that the cytokinin levels of the IAA-overproducing line are reduced due to increased cytokinin metabolism. The cytokinin data of the 35S:*iaaM* x 35:*iaaH*-plants could be explained by a lower cytokinin biosynthesis rate, but no experimental evidence is available yet to support this hypothesis.

16.3 IAA biosynthesis in tobacco during vegetative growth

To advance the understanding of IAA-biosynthesis in tobacco, experiments with labelled tracers were performed in

protoplasts and intact shoot tissue of wiltype or 35S*iaaM* x 35S*iaaH*-transgenic tobacco (SR1) plants(16.1). The aim was to estimate the contribution of the tryptophan (Trp) -independent IAA-biosynthesis pathway (see 3.) from measurements of the rate of IAA-turnover and the rate of Trp-dependent IAA-biosynthesis. Experiments were performed during a period of development when constant IAA-level was found in the apical region of both sets of plant. The strategy was to calculate the rate of IAA-synthesis through the Trp-independent pathway as the difference between the rate of IAA turnover and the contribution of the Trp-dependent pathway (**VI**). Based on the data obtained from the experiments with wildtype protoplasts, the Trp-independent pathway supplied 80% of the IAA synthesised *de-novo* during vegetative growth.

It is also possible that the 20% of the total IAA biosynthesis assigned to the Trp-dependent pathway is an overestimation, if exogenous Trp-feedings give rise to artificially high conversion rates, as shown to be the case in *Lemna gibba* due to improper compartmentalisation of the tracer (Rapparini *et al.* 1999).

In the 35S:*iaaM* x35S:*iaaH*-protoplasts, higher rates of IAA-turnover and Trp-dependent IAA-biosynthesis were found than in wildtype protoplasts. However, the deduced rates of Trp-independent IAA biosynthesis were comparable between the two types of protoplasts, indicating that the activity of this pathway is not affected by the increased IAA pool size in the transgenic line.

The experiments performed using intact plant tissues essentially failed. Inconsistent data on [$^{13}C_6$]IAA-turnover indicated that the tracer was non-homogenously distributed in the intact plant tissue. The wide experimental variation in the experiments was in marked contrast to the precision in the protoplast experiments. The conclusion is that this type of experiment should, if possible, be performed in experimental systems like protoplasts or *Lemna gibba* (Tam *et*

17. Transgenic Tobacco Plants Expressing the *ipt*, *iaaM* and *iaaH*-Genes

17.1 Crosses between *ipt*- and *iaaM* x *iaaH*-expressing parental lines

By crossing *ipt*-transgenic tobacco plants with *iaaM*-, *iaaH*-transgenic lines, plants expressing both auxin and cytokinin biosynthesis genes were obtained. In the crosses, increased levels of both cytokinin and auxin were expected, thus limiting the changes in auxin/cytokinin-ratios. Under such conditions, a phenotype close to the wildtype would be predicted by the hypothesis that the cytokinin:auxin ratio is critical in plant development, suggested by Skoog and Miller (1957).

Staffan Eklöf previously produced crosses from five transgenic parental lines:

1. "P3:*ipt*" (IV).
2. "P9:*ipt*" (IV).
3. "35S:*iaaM*, 35S:*iaaH*": a W38 line with both auxin biosynthesis genes driven by the CaMV 35S RNA promoter (Sitbon 1992), (see also 16.1).

Line	'Cytokinin traits'*	'Auxin traits'†
P3, *ipt* x 1'p-*iaaM* + 2'p-*iaaH*	+++	+
P3, *ipt* x 35S-*iaaM* x 35S-*iaaH*	+++	++
P3, *ipt* x 35S-*iaaM* + *iaaH* cv. SR1	+++	+++
P9, *ipt* x 1'p-*iaaM* + 2'p-*iaaH*	+	+
P9, *ipt* x 35S-*iaaM* x 35S-*iaaH*	+	++
P3, *ipt*	++++	-
P9, *ipt*	+	-
1'p-*iaaM* + 2'p-*iaaH*	-	++
35S-*iaaM* x 35S-*iaaH*	-	+++
35S-*iaaM* + *iaaH* cv. SR1	-	++++

Table 1. Lines resulting from crosses of cytokinin-overproducing *ipt* plants and auxin-overproducing *iaaM* + *iaaH* plants. The strength of the phenotypic traits associated with the two hormones are indicated in a four graded scale, where + corresponds to a barely detectable phenotype and ++++ to the most extreme one.
* Release of axillary buds, wrinkled and rounded leaves, reduced root growth and shortened internodes.
† Adventitious root formation, downwards bending of leaves, erected leaf blades, leaf edge rolling and stiff, brittle leaves.

4. "2'*:iaaM*, 1'*:iaaH*": a W38 line containing the *A. tumefaciens* IAA biosynthesis genes expressed from the bi-directional 1'2' T_R-DNA mannopine synthase promoter (Velten and Schell 1985) (Sitbon 1992).
5. "35S*:iaaM, iaaH* cv SR1": a doubly transformed *N. tabacum* L. cv. SR1 line that carries the *iaaM* gene under the control of the 35S promoter and the *iaaH* gene under the control of its natural promoter (Sitbon *et al.* 1992).

17.2 Morphological consequences

Surprisingly, all the phenotypic traits of the parental lines were also present in the crosses, giving rise to a mixed phenotype (Table1)(**VII**). The crosses showed reduced auxin-associated traits and also slightly weaker cytokinin-associated traits than the respective parental lines, presumably due to the antagonistic interactions between auxins and cytokinins. The fact that the dual phenotype was not restricted to a single cross demonstrated that it was generally applicable and not linked to the *ipt*-, *iaaH*- and *iaaM*-expression pattern in a single plant line. The tissue specificity

of the P3- and P9- promoters is largely unknown and it has been suggested that a spatial variation in the *ipt*-expression could cause the dual phenotypes. However, the fact that there are no qualitative differences between the P3- and P9-crosses argues against this hypothesis. A correlation between the strength of the auxin- and cytokinin- phenotypical traits in the crosses and the parental lines is also present, indicating a hormone dosage effect.

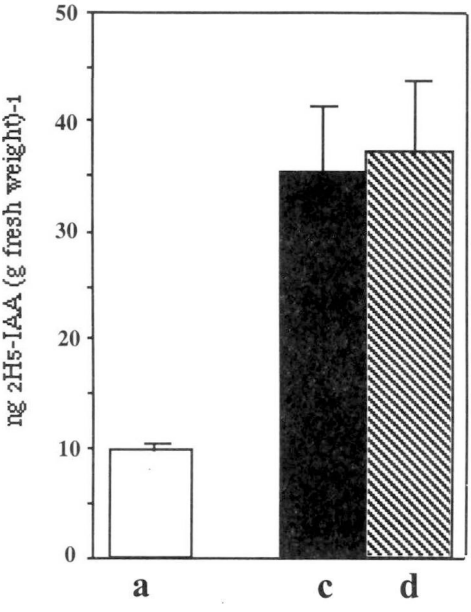

Fig. 3. Level of $^2H_5$-IAA synthesised from $^2H_5$-Trp fed to leaf-discs excised from the leaf immediately below the leaves used for hormone and northern analysis. +SD is indicated on the bars.
(a); wt, **(c)**; P3:*ipt* x 35S:*iaaM*, 35S:*iaaH* (cross), **(d)**; 35S:*iaaM*, 35S:*iaaH* (*iaa*-parental)

17.3 Auxin and cytokinin analysis

The P3:*ipt* x 35S*iaaH*, 35S*iaaM* line was chosen for further examination and the auxin and cytokinin contents of the young leaves were quantified (**VII**). The measurements showed, surprisingly, that the ZR+ZRMP and the IAA levels of the cross were not significantly different from those of wildtype plants.

Thus, the very high IAA content in the *iaaH*, *iaaM*-expressing parental line was downregulated by the expression of the *ipt*-gene and the increased size of the ZR+ZRMP-pool in the *ipt*-expressing line was downregulated by the introduction of the IAA-biosynthesis genes.

This dramatic reduction in hormone levels is consistent with the role of cytokinins as downregulators of IAA and vice versa (**V**). Our previous experiments showed a reduction of the IAA-biosynthesis rate in the P3 cytokinin-overproducing plants, compared to wildtype plants (15.4) and that the lower cytokinin levels, found in the 35S*iaaM* x 35S*iaaH*, IAA-overproducing plants (16.2), possibly indicated an IAA-effect on the cytokinin biosynthesis rate. But in the cross the main biosynthetic activity of both auxins and cytokinins is mediated via the bacterial pathways, and plant homeostatic mechanisms are not expected to regulate their activity. Consistent with this expectation, no reduction in the level of expression of the bacterial IAA-biosynthesis pathway (*iaaM*, *iaaH*) was detected in the cross compared to the IAA-overproducing parental line (**VII**). To verify the expression data, plants from the IAA-overproducing parental line, the cross and wildtype were fed with [$^2H_5$]Trp and the conversion to [$^2H_5$]IAA was monitored by mass spectrometry. No significant difference in conversion capacity was found between the IAA-parental line and the cross, verifying that the expression level of the bacterial genes was maintained in the cross (Fig. 13).

A slightly lower level of expression of the *ipt*-gene was detected, than in the cytokinin-overproducing parental line, and this could partly explain the lower cytokinin levels in the cross. It is also likely that a higher turnover of both auxins and cytokinins occurs in the cross. However, the only data that support this hypothesis relate to conjugated IAA contents in the cross (Fig. 14), showing that the high levels found in the IAA-overproducing parental line (Sitbon *et al.* were maintained in the cross.

17.4 A potential model of explaination

Spatial differences in the auxin- and cytokinin- contents, leading to a wildtype-like average for the sampled tissue, is a potential explanation why no alterations in active hormone levels were detected in our study. Spatial variations in the auxin and cytokinin levels, due to differences in the auxin and cytokinin biosynthesis capacities, combined with variations in the metabolic activities, could also explain why cytokinin associated traits develop close to auxin associated traits. In a complex system of mixed cytokinin- and auxin- gradients, inductive conditions could arise to release a lateral shoot at one point, while markedly different conditions nearby could promote adventitious-root formation.

The data obtained from the iaa x *ipt*-cross does not really fit in the auxin/cytokinin ratio-hypothesis suggested by Skoog and Miller (1957). However, it is still possible that the conditions in target tissues or individual cells satisfy this model. Future studies of hormone levels in single cells or in small cell clusters may bring knowledge about the nature of this cytokinin-auxin interaction.

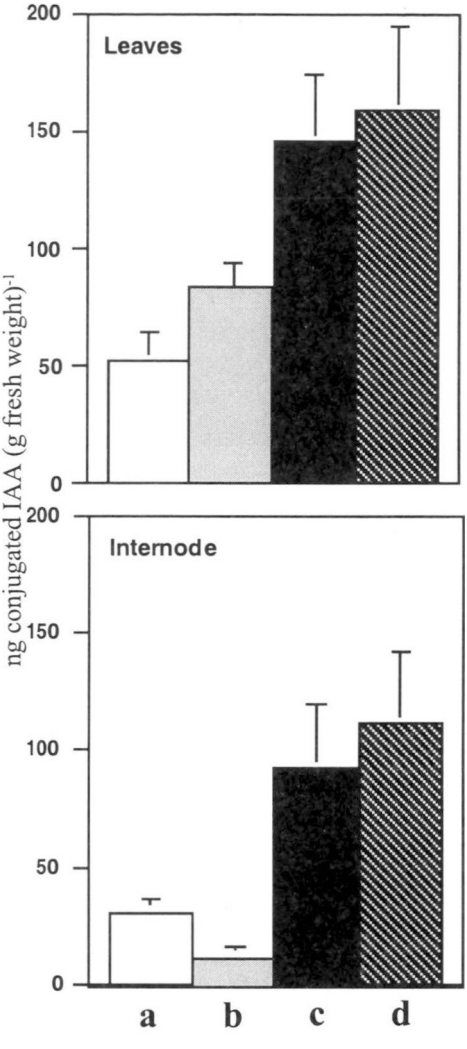

Fig. 5. Levels of conjugated IAA measured in extracts isolated from young leaves and the 4th internode from the base 8 weeks after germination. +SD is indicated on the bars.
(a); wt
(b); P3*:ipt* (*ipt* parental)
(c); P3*:ipt* x 35S*:iaaM*, 35S*:iaaH* (cross)
(d); 35S*:iaaM*, 35S*:iaaH* (*iaa* parental)

CONCLUSIONS

This thesis mainly describes the development of new tools for cytokinin analysis and the application of these techniques in investigations concerning cytokinin physiology. It is concluded that:

- Derivatization of cytokinins can dramatically improve the sensitivity of their analysis by FAB-mass spectrometry.
- Derivatization of cytokinins enriches their mass spectra, thereby increasing the qualitative information.
- *In-vivo* deuterium incorporation provides an excellent tool for analysis of cytokinin biosynthesis.
- An alternative, iPAMP-independent pathway is the main route for bacterial isopentenyltransferase-mediated cytokinin biosynthesis.
- The alternative, iPAMP-independent cytokinin biosynthesis pathway exists as an endogenous pathway in *Arabidopsis*.
- Moderate cytokinin overproduction in tobacco is not associated with a significant increase in cytokinin oxidase activity.
- Cytokinin-overproduction reduces IAA levels by reducing the IAA-biosynthesis rate.
- IAA-overproduction reduces cytokinin contents, but not by inducing cytokinin oxidase activity.
- IAA-biosynthesis in tobacco is mainly mediated by the tryptophan-independent pathway
- Coexpression of the *ipt*, *iaaM* and *iaaH*-genes gives rise to a mixture of auxin- and cytokinin- associated phenotypes, but is not associated with altered hormone levels.

The development of new analytical tools is essential for scientific advances, since progress of this kind enables new types of experiments to be designed. The new techniques developed for cytokinin analysis described in this thesis allowed us to test the established model for cytokinin biosynthesis, and provided evidence for an alternative pathway. The methods could be used for a number of related experiments in the future in order to investigate the localisation and regulation of cytokinin biosynthesis in plants.

Auxin/cytokinin interactions *in planta* were investigated in order to elucidate the mechanisms involved. The initial work suggested that absolute levels could be as important as the auxin/cytokinin-ratios, but in the transgenic tobacco plants expressing both heterologous cytokinin and auxin biosynthesis genes, a surprisingly complex situation was encountered. Neither the hormone ratios nor the absolute hormone levels could explain the phenotype of these plants. To test the models, analysis of hormone levels in specific target tissues is required, and until this is achieved key questions about cytokinin / auxin-interactions may remain unresolved.

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