Cytokinins in *Arabidopsis*, Tools, Pathways and Interaction with Auxin

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Abstract

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Cytokinins are plant hormones, and their relative abundance in proportion to auxin determines whether shoots or roots are formed in callus tissue. The cytokinin to auxin ratio is also believed to regulate apical dominance in plants. The suggested biosynthetic route for cytokinin involves formation of the nucleotide iPMP as the first metabolite synthesized, which is then subsequently hydroxylated to form the nucleotide ZMP. In the work underlying this thesis the potential role of an iPMP-independent biosynthetic route, with direct formation of the nucleotide ZMP was explored. Using an inducible IPT line of *Arabidopsis*, and *in vivo* deuterium labelling coupled to analysis with LC-MS/MS, we concluded that ZMP could indeed be formed independently of iPMP. In a similar experiment with wild type *Arabidopsis*, ZMP was also formed independently of iPMP. Furthermore, the two plant hormones cytokinin and auxin have been shown to regulate each other's metabolism. In experiments using the IAA analogue NAA, a very rapid decrease in the biosynthesis and pool size of cytokinins was observed. The effect of NAA was mediated through the iPMP-independent pathway. A negative effect of cytokinin on auxin biosynthesis and pool size was also detected. This regulation was found to be slower, and probably related to alterations in development. Arabidopsis and tobacco leaves and roots were separately incubated in media containing $^{2}H_{2}O$. Cytokinin biosynthesis capacity was detected in all tissues, and small leaves seemed to possess the highest *de novo* biosynthesis capability. An *Arabidopsis* mutant defective in production of the ADK enzyme responsible for conversion of adenosine to adenosine 5´monophosphate and the corresponding cytokinins was found to contain elevated levels of cytokinins, while its IAA levels were lower than in wild type. The increased cytokinin content was due to increased cytokinin biosynthesis. Finally, a LC-MS/MS method capable of quantifying central cytokinin metabolites in sample sizes <50mg FW including bases, ribosides and intact nucleotides at a rate of 70 samples/day was developed.

Keywords: *cytokinin, auxin, biosynthesis, Arabidopsis thaliana, liquid chromatography, ESI, MS/MS, interaction, development, plant hormones*

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I stunder av,

Högmod eller Svårmod
Hybris eller Ödmjukhet

har jag funnit ro i följande uppmuntrande ord signerade G.S.

”Du skall veta Anders, jag kan få en apa genom forskarutbildningen”

Contents

Appendix

List of Papers

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

I. Åstot, C., Dolezal, K., Nordström, A., Wang, Q., Kunkel, T., Moritz, T., Chua, N-H. and Sandberg, G. (2000) An alternative cytokinin biosynthesis pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 97, 14778-14783.

II. Nordström, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Åstot, C., Dolezal, K. and Sandberg, G. (2004) Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin-cytokinin-regulated development. *Proceedings of the National Academy of Sciences of the United States of America.* 101, 8039-8044.

III. Nordström, A., Tarkowski, P., Tarkowska, D., Dolezal, K., Åstot, C., Sandberg, G. and Moritz, T. (2004) Derivatization for LC-Electrospray Ionization-MS: A tool for improving reversed-phase separation and ESI responses of bases, ribosides, and intact nucleotides. *Analytical Chemistry*. 76, 2869-2877.

IV. Para, A., Nordström, A., Wilson, K., Moffat, B.A., Sandberg, G. and Sundås-Larsson, A. (2004) Disruption of the *ADK1* gene causes meristem distortion and a cytokinin overproduction phenotype in *Arabidopsis thaliana*. *Manuscript.*

Also published by the author but not included in the thesis:

Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M. and Moritz, T. (2004) Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of Arabidopsis thaliana samples in metabolomic studies with gas chromatography/mass spectrometry. *Analytical Biochemistry*. 331, 283-295.

Jonsson, P., Gullberg, J., Nordström, A., Kusano, M., Kowalczyk, M., Sjöström, M. and Moritz, T. (2004) A strategy for identifying differences in large series of metabolomic samples analyzed by GC/MS. *Analytical Chemistry*. 76, 1738-1745.

Viklund, C., Nordström, A., Irgum, K., Svec, F. and Frechet, J.M.J. (2001) Preparation of porous poly(styrene-*co*-divinylbenzene) monoliths with controlled pore size distributions initiated by stable free radicals and their pore surface functionalization by grafting. *Macromolecules*. 34, 4361-4369.

Background

Introduction

Multi-cellular organisms need signalling pathways to co-ordinate responses within the system. This enables each organism to proceed through the series of events that takes place during its life cycle. The signalling system reacts to both intrinsic and external factors, integrates the cues and initiates functional responses. In plants, several compounds are believed to be parts of either paracrine or long-distance signalling pathways. Some of these are referred to as “plant hormones” or “growth regulators”. Cytokinins and auxins are two classes of compounds that act as plant hormones.

This thesis deals with the biosynthesis of cytokinins and the interaction between cytokinins and auxin at the biosynthetic level. It seems logical to study these two compounds in conjunction with each other, since they have proven to regulate developmental processes in both antagonistic and synergistic manners. Moreover, their discovery can also be traced to the same group of researchers.

Folke Skoog (1908-2001) was working in the early 1930´s as an undergraduate/graduate student in the laboratories of Frits Went and Kenneth Thimann at the California Institute of Technology. He participated in the work leading to the discovery of IAA as a growth substance (Thimann & Koepfli, 1935). He and his collaborators found that material promoting growth in oat coleoptiles could be obtained from terminal buds of beans by allowing it to diffuse into agar blocks. Furthermore, when excised terminal buds of beans were placed directly on cut surfaces of decapitated oat plants, inhibition of lateral buds could be maintained. Auxin was the factor that inhibited growth of the lateral buds. They suspected that a second factor was involved in the process. In the mid 1950´s, Miller and Skoog described the first cytokinin, kinetin (Fig. 1), which they obtained from heated DNA (Miller & Skoog, 1955; Miller *et al.*, 1956). Although this was not an endogenous cytokinin, it displayed the ability to induce cell division and shoot formation from callus growing in a cell culture in concert with IAA (Skoog & Miller, 1957). The cited authors demonstrated that the ratio of kinetin to auxin determined shoot or root growth from a tobacco callus. If the ratio was high, the callus formed shoots and if the ratio was low, the callus formed root tissue. The conclusion was that the cytokinin to auxin balance is involved in organogenesis. However, it was not until 1958 that Thimann´s group showed that the “other factor” was a cytokinin and demonstrated its antagonistic effect to auxin (Wickson & Thimann, 1958). They excised stem parts and placed them in a growth medium. In a control (just medium) the lateral buds were released. If auxin was added this was inhibited. If both auxin and kinetin were added, the buds were released, demonstrating the antagonistic effect of these two compounds (Wickson & Thimann, 1958).

The name cytokinin derives from cytokinesis (cell division) and a cytokinin is now defined as “A compound that in the presence of auxin induces cell division in a suitable assay material grown on a defined medium” (Shaw, 1994).

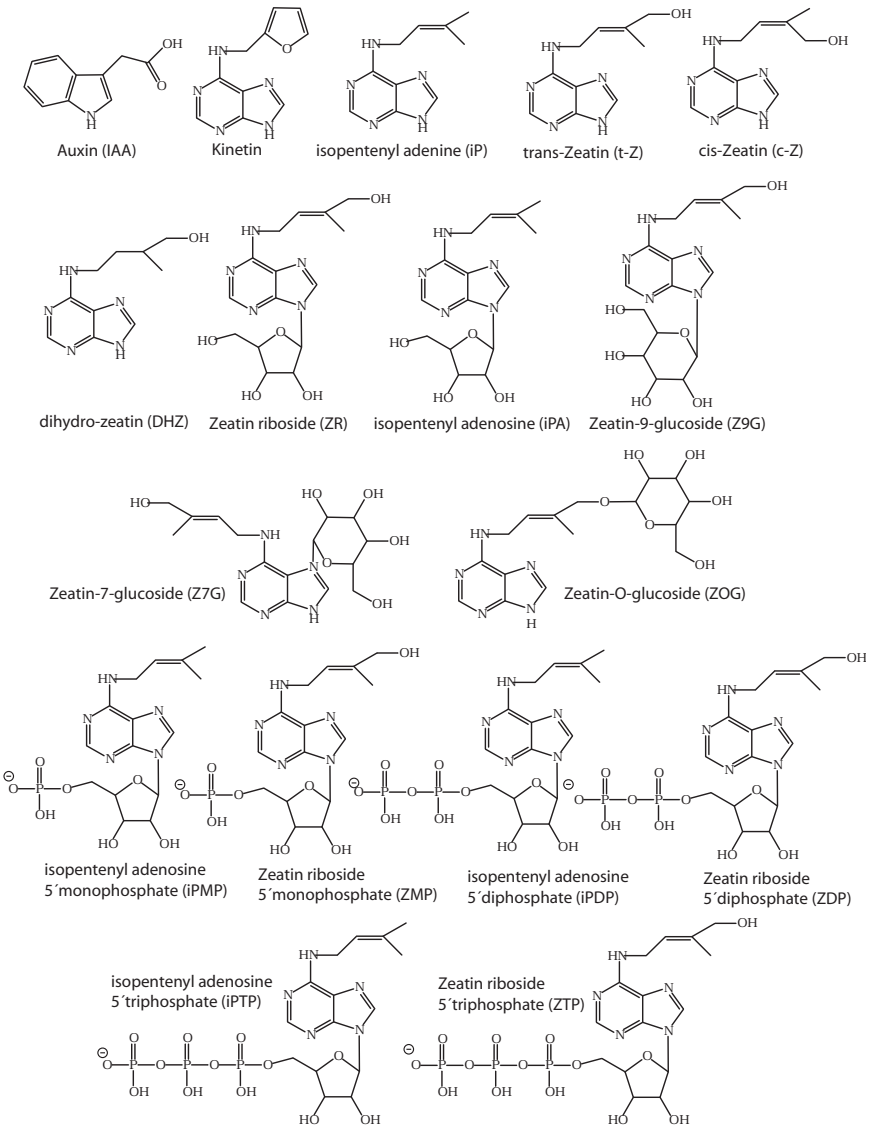

Figure 1. Selected cytokinin and Auxin metabolites

The first naturally occurring cytokinin that was identified was *trans*-6-(4-hydroxy-3-methylbut-2-enylamino)-purine (Letham, 1963; Letham, *et al*., 1964). The compound was named Zeatin (Fig. 1) after the plant *Zea mays* in which it was found. Zeatin was present at a very low concentration. Letham only obtained 1 mg of Zeatin from 70 kg of tissue! (Letham *et al*., 1964). Several naturally occurring cytokinins (as well as synthetic analogues) have been identified since then. The naturally occurring cytokinins are all adenines, substituted at N^6 with an isoprenoid or aromatic side chain some of which are displayed in Fig. 1. Cytokinins have

been found to play a part in many aspects of plant growth and development either antagonistically/synergistically with other plant growth regulators or by themselves. In the last few years, molecular evidence for endogenous cytokinin biosynthesis in plants (Kakimoto, 2001; Takei *et al*., 2001a), and their ability to perceive cytokinins, via the identification of cytokinin receptors, has been obtained (Inoue *et al*., 2001). Cytokinins have thus been definitively established as endogenously produced signalling compounds. For reasons of convenience, throughout this thesis I will refer to cytokinins as the naturally occurring, N^6 isoprenoid-substituted adenines.

Objectives

The main objectives of the work underlying this thesis were to investigate how cytokinins are synthesised *de novo* (Paper **I**) and how cytokinin and auxin regulate each other's metabolism (Paper **II**). Furthermore, we wanted to investigate potential sites of cytokinin biosynthesis (Paper **II**). The need for high-throughput quantification methods, capable of analysing cytokinins in sample sizes (< 100 mg FW) which are potentially more relevant for physiological studies than the large samples used in previous investigations was addressed in Paper **III**. How cytokinin-auxin homeostasis is altered in an *Arabidopsis* mutant defective in central cytokinin metabolism was examined in Paper **IV**.

Cytokinin Biosynthesis

There have been three main views on cytokinin synthesis, holding that:

A: They are not endogenously produced by plants, but rather produced by bacteria, and assimilated by plants in a symbiotic fashion (Holland, 1997).
B: They can be synthesised indirectly via plant tRNA.
C: They can be synthesised directly, *de novo*, by plants.

A: The first view has been proved wrong, at least in the sense that cytokinins *cannot* be synthesised by plants. Genes encoding enzymes for *de novo* cytokinin synthesis have been found in *Arabidopsis* (Kakimoto, 2001; Takei *et al*., 2001a). Moreover, aseptically grown plants have also demonstrated *de novo* cytokinin biosynthesis.

B: Regarding the second view, cytokinins were found to be located adjacent to the 3´-end of the anticodon in tRNA obtained from yeast (Zachau *et al*., 1966; *Bieman et al*., 1966). Since then, cytokinin-active ribonucleosides have been reported in tRNAs from virtually all organisms tested. It has been suggested that the action of cytokinins might be mediated through the function of cytokinins in tRNA on protein synthesis. Six different cytokinins have been discovered in plant tRNA, including cis/trans-ZR and cis/trans-iPA, of which cis-ZR seems to be the most abundant (Taller, 1994). Recently, a gene coding for a tRNA isopentenyltransferase enzyme was cloned in *Arabidopsis* (Golovko *et al*., 2002). In incorporation studies of radioactive adenine in intact bean root, most of the cytokinin subsequently detected appeared to originate from RNA hydrolysis (Maaβ & Klämbt, 1981). The fact that cis-type cytokinins are the most abundant cytokinins in tRNA has raised the question

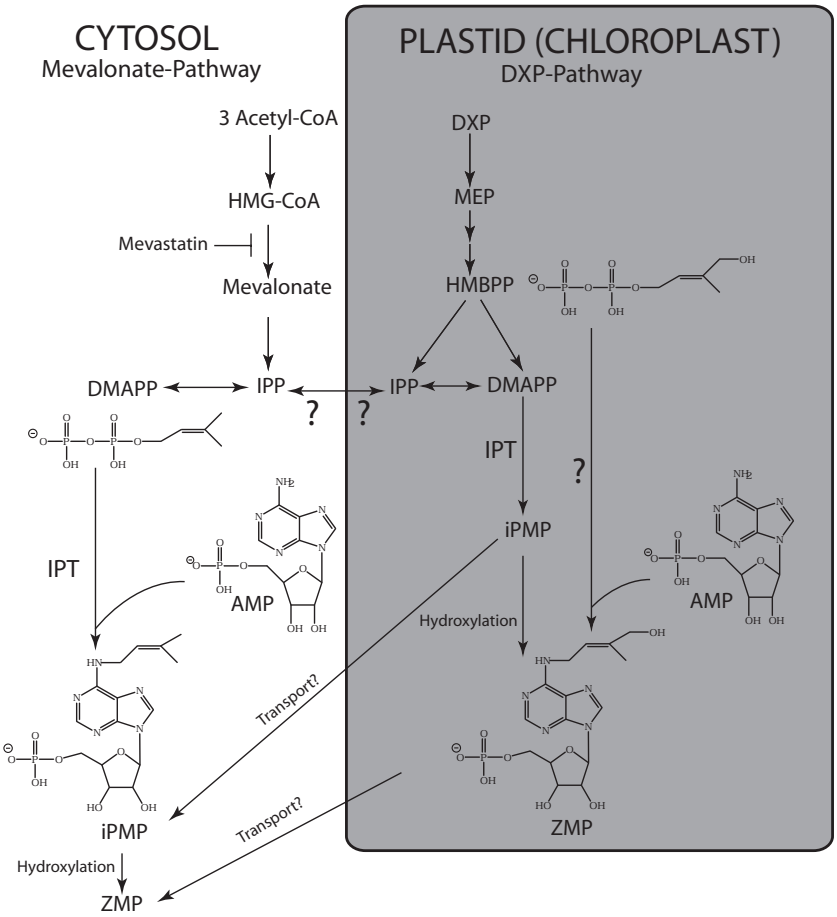

Figure 2. Proposed compartmentalization of cytokinin metabolism in plant cells. In the cytosolic Mevalonate (MVA) pathway, which can be inhibited by mevastatin, isopentenyl transferase, IPT, catalyses the transfer of DMAPP to AMP/ADP/ATP leading to the formation of iPMP. In the plastid localized 1-Deoxy-D-xylulose 5-phosphate- (DXP) pathway, also referred to as the Methyl-erythritol-phosphate (MEP) pathway, the cytokinin ZMP is formed by the addition of HMBPP to AMP/ADP/ATP.

if tRNA actually can act as a source of cytokinins, since cis-type cytokinins are biologically inactive (Murai, 1994). The occurrence of cis/trans isomerases (Bassil *et al*., 1993), might partially explain how biologically inactive cis-cytokinins, derived from tRNA are modified to become active cytokinins. However, tRNAs are estimated to account for at most 40% of the cytokinin biosynthesis when calculated from the tRNA turnover rate based on radioactive feeding experiments (Barnes *et al*., 1980), and it is generally assumed that tRNAs play only a minor role, if any, as sources of cytokinin precursors (Barnes *et al*., 1980; Klämbt, 1992).

C: The generally accepted view has been that plants do synthesise cytokinins endogenously. *Dictyostelium discoideum* (slime mold) gave the first evidence of direct *de novo* cytokinin biosynthesis from AMP and an isoprenoid side chain. A partially purified enzyme from this organism catalysed the transfer of the isopentenyl moiety from dimethylallyldiphosphate (DMAPP) to AMP as illustrated on the cytosolic side

in figure 2. This was referred to as DMAPP:AMP isopentenyltransferase (iptase) activity and the enzyme was referred to as an isopentenyl transferase IPT (Taya *et al*., 1978). The same activity was detected in cell-free extracts of cytokinin-autotrophic tobacco tissue culture (Chen & Melitz, 1979). The gene that encoded a similar IPT was cloned in *Agrobacterium tumefaciens* (bacteria) from the Ti-plasmid (Barry *et al*., 1984; Akiyoshi *et al*., 1984). Activity of IPT from partially purified corn kernels was demonstrated by Reinecke *et al*. (1991), who further demonstrated that the enzymatic activity decreased during *Zea mays* kernel development (Reinecke *et al*., 1992). Similarly, IPT activity was described by Blackwell & Horgan (1994) from crude extracts of *Zea mays* kernels. The IPT enzyme is very unstable and it has proven to be difficult to purify to homogeneity. Another cytokinin biosynthetic gene, *TZS* (trans-Zeatin synthase), is present in the virulence region of the Ti-plasmid (that is, it is not located in the T-DNA which is actually transferred to the plants at infection) in some strains of *Agrobacterium tumefaciens* (Kaiss-Chapman & Morris, 1977). This gene, when cloned and expressed in *Escherichia coli* resulted in the secretion of Zeatin (Beaty *et al*., 1986). The conclusion from all these findings is that the initial step in cytokinin biosynthesis is the catalytic transfer by an IPT-enzyme of DMAPP to AMP (Fig. 2), producing iPMP as the first cytokinin metabolite.

Kakimoto explored the *Arabidopsis* genome database and searched for genes with similarity to the *Agrobacterium IPT* sequence, finding nine putative Arabidopsis *IPT* genes which were named *AtIPT1* to *AtIPT9* (Kakimoto, 2001). Independently, another Japanese research group made the same discovery (Takei *et al*., 2001a). In an attempt to further characterise these putative cytokinin biosynthesis genes, Kakimoto over-expressed the AtIPT4 gene in *Arabidopsis* calli. The calli were able to regenerate shoots even in the absence of cytokinins in analogy with the experiments performed by Skoog and Miller (1957). This demonstrated the ability of *AtIPT4* to synthesise cytokinins (Kakimoto, 2001). In a second experiment the *ATIPT4* gene was expressed in *E. coli*. In kinetic studies subsequently performed on the gene product, Kakimoto found that the *AtIPT4* was a DMAPP:ATP/ADP isopentenyl transferase rather than a DMAPP:AMP isopentenyl transferase. This implies that the first cytokinin metabolites formed could be iPTP or iPDP rather than iPMP (Fig. 3) (Kakimoto, 2001). Takei and co-workers expressed *AtIPT1* to *ATIPT8* in *E. coli* and detected Z and iP in the culture medium (Takei *et al*., 2001a). The relative abundance of Z and iP varied somewhat, and for AtIPT1, 4, 7, 8, the Z/iP ratio was higher than for AtIPT3, 5, 6 (Takei *et al.*, 2001a). Further, *in vitro* characterisation of the purified AtIPT1 gene product, revealed DMAPP:AMP isopentenyl transferase activity (Takei *et al*., 2001a).

The finding by Kakimoto that *AtIPT4* had much higher substrate specificity for ATP and ADP than AMP (Kakimoto, 2001) is rather intriguing. As already mentioned, this implies that early cytokinin metabolites might be di- or tri-phosphates rather than mono-phosphates (Fig. 3). The existence of such cytokinin metabolites have been suspected for quite some time (Miller, 1967). For instance, Laloue *et al.* (1974) demonstrated that these metabolites could be formed in plants. They fed cytokinin-dependent tobacco cell culture with radiolabelled iPA and demonstrated that iPDP and iPTP were formed (Laloue *et al*., 1974). These two metabolites accounted for 77% of the extracted radioactivity. In order to minimise potential interference from

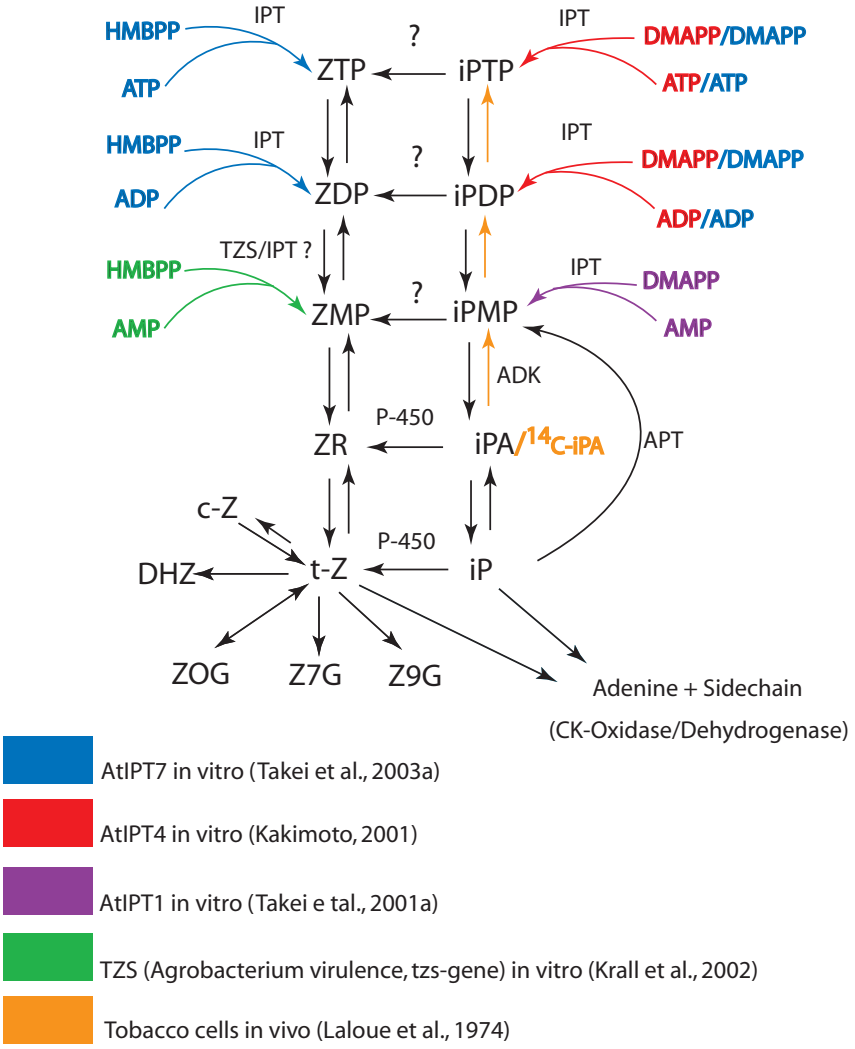

Figure 3. Proposed cytokinin biosynthetic scheme (not showing all known conjugations).

other labelled purines present in the extraction mix, they also demonstrated that di- and tri-phosphate metabolites were formed from labelled BAP (benzyl amino purine) (Laloue *et al*., 1974). The formation of di- and tri-phosphate cytokinin metabolites in feeding experiments with radiolabelled compounds has since been shown in tobacco cell culture (Laloue *et al*., 1977a; Laloue & Pethe, 1982) and cell cultures of the moss *Physcomitrella patens* (Schwartzenberg *et al*., 2003).

The proposed cytokinin biosynthesis pathway is displayed in figure 3. A factor of further importance is the finding of direct *de novo* biosynthesis of ZMP (Åstot *el al*., 2000a). Over-expression of the *Agrobacterium IPT* gene in tobacco (Redig *et al*., 1996a; Faiss *et al*., 1997) and *Arabidopsis* (Åstot *et al*., 2000a) resulted in large increases in zeatin-type cytokinins, but only modest increases in iP-type cytokinins. This had earlier been attributed to rapid metabolism of iPMP to its hydroxylated (zeatin-type) form, ZMP (Palni & Horgan, 1983). However, with

in vivo deuterium labelling and double tracer feeding it became evident that there is an iPMP-independent pathway for the synthesis of ZMP (Åstot *et al*., 2000a). The proposed iPMP-independent pathway would use 4-hydroxy-3-methyl-2-(E)-butenyldiphosphate (HMBPP), rather than DMAPP as the side chain precursor (Fig. 2+3). Plants have two possible biosynthesis pathways for the isoprenoid side chain, the DXP (1-Deoxy-D-xylulose 5-phosphate) pathway in plastids (chloroplasts) and the MVA (Mevalonate) pathway in the cytosol (Lichtenthaler, 1999; Rohmer, 2003) (Fig.2). It should be mentioned that the DXP-pathway is also referred to as the MEP (methylerythritolphosphate) pathway. Both pathways can supply DMAPP and IPP, but HMBPP has only been found to be an intermediate in the plastid localised DXP-pathway (Hecht *et al*., 2001) (Fig. 2).

In an attempt to investigate the possible origins of the isoprenoid side chains of cytokinins, Kasahara and co-workers utilised ^{13}C-substrate feeding and a two-way approach involving both Arabidopsis mutants and an enzyme inhibitor (Kasahara *et al*., 2004). An *Arabidopsis* mutant, *cla1*, which is defective in the 1-deoxy-D-xylulose-5-phsophate synthase (DXP synthase) of the DXP pathway (Estevez *et al*., 2000) was used for studying ^{13}C-labelling patterns generated when the DXP pathway is defective. Mevastatin, which selectively blocks the HMG coA reductase of the MVA (Mevalonic acid) pathway (Fig 2) was used to investigate the labelling pattern when the MVA-pathway was blocked (Kasahara *et al*., 2004). The cited authors demonstrated that trans-Z and iP had mainly plastid origins, whereas the cis-Z side chain had a cytosolic origin (Kasahara *et al*., 2004). A finding that further strengthens the hypothesis that cytokinin isoprenoid side chains are of plastid (DXP-pathway origin) was the discovery that cytokinin levels were not affected in a mutant with a dysfunctional *hmg1* gene, which encodes the HMG reductase of the cytosolic pathway (Suzuki *et al*., 2004). Moreover, it was previously demonstrated that expression of the *Agobacterium TZS* gene in *E. coli* results in secretion of Z (Beaty *et al*., 1986). The gene encoding this enzyme was cloned, expressed and the enzyme was purified and studied *in vitro* by Krall and co-workers. With the radioactively labelled substrate HMBPP and AMP they demonstrated conversion to ZMP (Krall *et al*., 2002). Takei and co-workers prepared Z- and iP-type cytokinin di- and tri-phosphates standards by expressing *AtIPT7* in *E. coli* and subsequently utilised *in vitro* reactions for the actual synthesis. They found that the reaction efficiency was lower when using HMBPP as a substrate than DMAPP, indicating that the gene product of *AtIPT7* has higher specificity for DMAPP (Takei *et al*., 2003a). This group also developed a method for separating these di- and tri-phosphates from plant material (Takei *et al*., 2003b). However, they did not demonstrate unambiguously the presence of the compounds *in planta*. In contrast, the above findings, which support *de novo* ZMP synthesis independent of iPMP biosynthesis, over-expression of *AtIPT8* in *Arabidopsis* led to an accumulation of iP-type cytokinins in a study by Sun *et al*. (2003).

Taken together, these findings show that plants have the ability to synthesise cytokinins endogenously. There seems to be two pathways for *de novo* cytokinin biosynthesis, but their relative importance is not fully understood.

Metabolism and Catabolism

A summarised version of some of the known cytokinin metabolic conversions is displayed in figure 3. Inter-conversions between cytokinin bases, ribosides and nucleotides are mediated by the same enzymes that convert their adenyl analogues (Mok & Martin, 1994). Generally these enzymes show higher affinity for the adenine compounds than for their cytokinin relatives, as exemplified in kinetic studies in *Arabidopsis* with adenosine phosphoribosyl transferase APT (Allen *et al*., 2002) and adenosine kinase (ADK) (Moffat *et al*., 2000). Other changes to the adenine structure are more specific for the adenine type cytokinins. These include glucolysation at the 3, 7 or 9 position at the purine ring by glucosyl transferase (Entsch *et al*., 1979). These metabolites are not subject to hydrolysis of the glucoside unit by β-glucosidase and are biologically inactive (Laloue, 1977b). They seem to be very stable conjugates and are considered to be permanently inactivated forms. There is conflicting evidence regarding their ability to serve as substrates for cytokinin oxidase (Schmülling *et al*., 2003). Conjugates of cytokinins may also occur with alanine at the 9 position, lupinic acid (Parker *et al*., 1975).

Modification to the side chain is more specific to cytokinins. A reductase that is highly specific for *trans*-Z has been isolated in *Phaseolus* (Martin *et al*., 1989) that is responsible for reduction of the isoprenoid side-chain double bond. The enzyme did not modify *cis*-Z, ZR or iP. Cytokinin oxidase does not seem to use DHZ as a substrate (Bilyeu *et al*., 2001), and hence the formation of dihydro-metabolites might be important in maintaining cytokinin homeostasis. Glycosylation of the isoprenoid side-chain hydroxyl group of Z seems to be a key process in the machinery that regulates the level of active cytokinins. Zeatin-O-glucoside, ZOG, and the corresponding Zeatinriboside-O-glucoside, ZROG, display low biological activity and are converted back to their active Z or ZR forms by β-glucosidases (Brzobohaty *et al*., 1993; Mok & Mok, 2001). The glycosylation is mediated by Zeatin-O-glycosyltransferase. The gene that encodes this enzyme, *ZOG1*, has been cloned in *phaseolus lunatus* (Martin *et al*., 1999). The enzyme responsible for O-glucosylation of *cis*-Z has also been identified in maize (Martin *et al*., 2001; Veach *et al*., 2003). Conversion of iP-type cytokinins (iP and iPA) to their hydroxylated Z-type counterparts is believed to be mediated by microsomal, mixed function oxidases, particularly cytochrome P-450 enzymes (Chen & Leisner, 1984). Activity of cytokinin *cis*-*trans* isomerases has been detected in extracts from *Phaseolus* (Bassil *et al*., 1993). This enzyme might participate in regulation of the pool of active cytokinins by converting less active *cis*-Z to active *trans*-Z. Incidentally, in maize, *cis*-Z seems to be an active cytokinin (Yonekura-Sakakibara, 2004). Therefore one might draw erroneous conclusions about cytokinin metabolism and its regulation, when comparing data from many different species.

Cytokinin oxidase catalyses the irreversible degradation of iP, Z and their corresponding ribosides in a single step by oxidative side-chain cleavage (Schmülling *et al*., 2003). The enzyme seems to be expressed from a family of seven genes in *Arabidopsis* (Schmülling *et al*., 2003). However, it has been demonstrated that the enzymes do not need molecular oxygen as an electron acceptor but rather use a flavin co-factor (Galuszka *et al*., 2001). This implies that the enzyme should be

named cytokinin dehydrogenase rather than cytokinin oxidase. Cytokinin oxidase/dehydrogenase activity is up-regulated by cytokinins (Dietrich *et al*., 1995; Motyka *et al*., 1996; Motyka *et al*., 2003). Predicted sub-cellular localizations derived from signal peptide analysis of the gene sequences include extra-cellular, cytosolic and plastid locations for the different gene products (Emanuelsson *et al*., 2000). One should be careful when interpreting such results, but experimental data have so far supported an extra-cellular localization since cytokinin oxidase/dehydrogenase activity has been found in the media of moss protoplast (Houba-Herin *et al*., 1999), yeast (Werner *et al*., 2001) and tobacco (Motyka *et al*., 2003) cultures. This is consistent with the finding that the CRE1-cytokinin receptor (Inoue *et al*., 2001) seems to be localised in the cell membrane, suggesting that some cytokinin signalling (at least) originates from the extra-cellular space, and thus regulation of the cytokinin content should be controlled, at least partially, by processes that occur outside of the cell wall. Recently a transgenic *Arabidopsis* was engineered to over-express cytokinin oxidase/dehydrogenase (Werner *et al*., 2003). Werner and co-workers found in fluorescently labelled fusion protein experiments that some cytokinin oxidases were targeted for the vacuole and the endoplasmatic reticulum (ER), supporting the hypothesis that some of the products of this gene family might be secreted from the cell (Werner *et al*., 2003). Expression studies using GUS-constructs revealed tissue-specific expression for the different genes in the cytokinin oxidase/dehydrogenase family (Werner *et al*., 2003). The transgenic plant displayed lowered levels of endogenous cytokinins, and would be highly suitable for investigating the significance of cytokinins *in planta*.

Taken together, these data suggest that cytokinin levels are regulated in plants at the biosynthetic level, by base to nucleotide/riboside conversions, irreversible inactivation to N-glucosides, reversible inactivation to O-glucosides and via permanent inactivation by oxidative cleavage of the side-chain by cytokinin oxidase/dehydrogenase.

Cytokinin Sites of Synthesis and Translocation

Cytokinins have been found in roots, stems, leaves, flowers, fruits and seeds, and they are probably present in every tissue in a living plant. This, however, does not necessarily imply that they are synthesised by every cell. A large set of data indicates that roots, especially root tips, are major sites of cytokinin biosynthesis (reviewed by Letham, 1994). During aseptic culture of maize roots by Van Staden & Smith (1978) cytokinins were released into the medium. In a similar experiment by Koda & Okazawa (1978) it was demonstrated that after growth of excised tomato root tips for seven days the cytokinin level in the media was eight times higher than in the root tissue. Studies on extracts of sunflower root apices 0-1 and 1-3 mm from the tip have found a 100-fold higher activity of cytokinins in the former location (Weiss & Vaadia, 1965). Chen and co-workers demonstrated incorporation of ^{14}C-adenine into cytokinins in pea and carrot roots when grown on media (Chen *et al*., 1985). Their results indicated that roots are the major source of cytokinin biosynthesis, but not the only one. Indirect evidence of root tip localised cytokinin biosynthesis has also been obtained, in studies involving, for instance, immunochemical staining (Sossountzov *et al*., 1988) and decapitation of root tips followed by observed

reductions in cytokinin contents (Feldman, 1979). Although it must be stated that some of these measurements lack the precision required by today's standards and consequently some of the evidence for root-localised cytokinin biosynthesis should be questioned. Some investigations have also pointed towards a possible role of apical plant parts in cytokinin biosynthesis. Incorporation of ^{14}C-adenine into cytokinin in apparently rootless tobacco plants suggests possible apical cytokinin synthesis (Chen & Petschow. 1978).

After identification of the cytokinin biosynthesis genes *AtIPT1* to *AtIPT9* (Kakimoto, 2001; Takei *et al*., 2001a), expression studies of these genes have been performed (Miyawaki *et al*., 2004). These investigators used beta-glucoronidase (GUS) fusion constructs with all of the AtIPT genes in *Arabidopsis*. They concluded that the genes were predominantly expressed in the following tissues: AtIPT1::GUS in xylem precursor cell files in the root tip, leaf axils, ovules and immature seeds, AtIPT3::GUS in phloem tissues, AtIPT4::GUS and AtIPT8::GUS in immature seeds with highest expression in the chalazal endosperm (CZE), a structure in the nutritious tissue that surrounds the embryo, AtIPT5::GUS in root primordia, root caps, upper parts of the young inflorescence and fruit abscission zones, and AtIPT7: :GUS in endodermis of the root elongation zone and trichomes (outgrowths) on young leaves. AtIPT2 and AtIPT9, which are responsible for tRNA isopentenyl transferases, were expressed ubiquitously (Miyawaki *et al*., 2004). These findings suggest that cytokinin biosynthetic capacities occur throughout the plant, and that many of the genes are expressed in different parts of the root structure.

Different sites of synthesis and action imply translocation. There are numerous demonstrations of cytokinin activity in xylem sap (reviewed by Letham, 1994). A gene family of high affinity transporters for adenine and purine derivatives in *Arabidopsis* has been identified (Gillissen *et al*., 2000; Bürkle *et al*., 2003). Expression studies of these genes have indicated that they have a role in retrieving root-synthesised cytokinins from the xylem and thus facilitating long-distance transport of cytokinins (Bürkle *et al*., 2003). Further evidence of xylem translocation of cytokinins was obtained by Takei and co-workers when studying the cytokinin response to nitrogen availability in maize. Upon addition of nitrogen to nitrogen-depleted plants, iPMP started to accumulate in the roots within an hour. This preceded the accumulation of ZMP and Z in the xylem sap. In the leaf tissue, Z started to accumulate 4 h after addition of nitrogen, and the elevated Z levels were maintained for at least 24 h (Takei *et al*., 2001b). Furthermore, in a subsequent investigation they also observed that the ZOG levels started to decrease in the root tissue upon nitrogen availability, suggesting increased conversion of inactive ZOG to active Z (Takei *et al*., 2002). The potential role of cytokinins in the phloem is not well understood. There are few reports, and only a few cytokinins have been identified with certainty: these include nucleotides (Vonk, 1978) and more recently Z and ZR were identified in phloem sap from the bean plant *Ricinus* (Kamboj *et al*., 1998). Translocation of cytokinins within the germinating seed is fairly well established. The incorporation of radioactively-labelled adenine into DHZR (Dihydrozeatin riboside) and DHZRMP (Dihydrozeatin riboside 5´monophosphate) has been demonstrated, and the subsequent polar movement of these compounds from embryo to emerging cotyledons in lupin seeds (Nandi *et al*., 1988; Nandi & Palni, 1989). Furthermore, excision of the embryonic axis by Munoz *et al*. (1990)

led to reduced cytokinin levels in the cotyledons. However, in the light of very recent discoveries, one must question these findings, at least as general conclusions. It appears that in *Arabidopsis*, no cytokinin receptors are expressed, either in the embryo or the developing seedling (Higuchi *et al*., 2004). Without any receptors, what would the purpose of cytokinin be as a signal? Indeed, there are studies that question whether cytokinins are long-distance signalling compounds, and rather propose a paracrine mode of action. Grafting experiments using tobacco rootstock with inducible cytokinin over-production and wild type shoots resulted in a 50-fold elevation of ZR in the roots 24 h after induction. However, no elevation of cytokinin in the shoots was detected (Faiss *et al*., 1997). To circumvent potential translocation problems due to grafting, Böhner used a dexamethasone-inducible/tetracycline-repressible expression system of the bacterial *IPT* gene in tobacco (Böhner & Gatz, 2001). When the *IPT* gene was induced in the tobacco plants, release of axillary buds was observed. Following this, tetracycline was applied locally to leaf-axils, causing repression of *IPT* expression. This immediately arrested the axillary bud outgrowth, and demonstrated that cytokinin produced elsewhere in the plant was not sufficient to release the bud outgrowth (Böhner & Gatz, 2001). These experiments raise questions about cytokinins as long-distance signalling compounds.

Cytokinin perception and signal transduction

Regardless of the possible role of cytokinins as long-distance or paracrine signalling substances, they are perceived and the signals are somehow relayed to gene expression and an effect is mediated. The first indication that histidine kinases might be involved in signal transduction of cytokinins came with the identification of the *cki1* mutant (Kakimoto, 1996). Over-expression of *CKI1* (*AHK1*) induced typical cytokinin responses independently of cytokinins. However, it later became evident that *CKI1* does not encode a receptor since it was constitutively active as a histidine kinase when expressed in *E. coli*, and not only in response to applied cytokinins. The discovery of the first true cytokinin receptor was made by three independent groups and designated *CRE1* by Inoue *et al*. (2001) or *AHK4* (Suzuki *et al*., 2001a; Ueguchi *et al*., 2001a). The *CRE1* (Inoue *et al*., 2001) gene was discovered by screening *Arabidopsis* calli for calli that were resistant to cytokinins. They found the cytokinin response1-1 gene (*cre1*), which was identical to *wol* (Scheres *et al*., 1995; Mähönen *et al*., 2000) and *AHK4* (Ueguchi *et al*., 2001a). The *CRE1* gene codes for a histidine kinase. To determine the molecular role of the gene, Inoue and co-workers used a mutant of *Saccaromyses cerevisiae* in which the only histidine kinase was disrupted (*sln1*). This mutant is lethal to yeast owing to the lack of phosphate-transfer. When CRE1 was introduced in *sln1*, rescue from the lethality only took place in the presence of cytokinins (Inoue *et al*., 2001). This experiment, coupled with the *cre1* mutant's insensitivity to cytokinins, clearly demonstrated the role of *CRE1* as a cytokinin receptor. It has now become apparent that cytokinin perception and signal transduction in *Arabidopsis* is mediated via a two-component system (Fig. 4), consisting of the histidine kinase and a response regulator. Most histidine kinases are trans-membrane receptors with a signal sensing domain in the extra-cellular space and a signal transducing domain in the cytoplasm. Response regulators have a receiver domain and often an output domain which, for example,

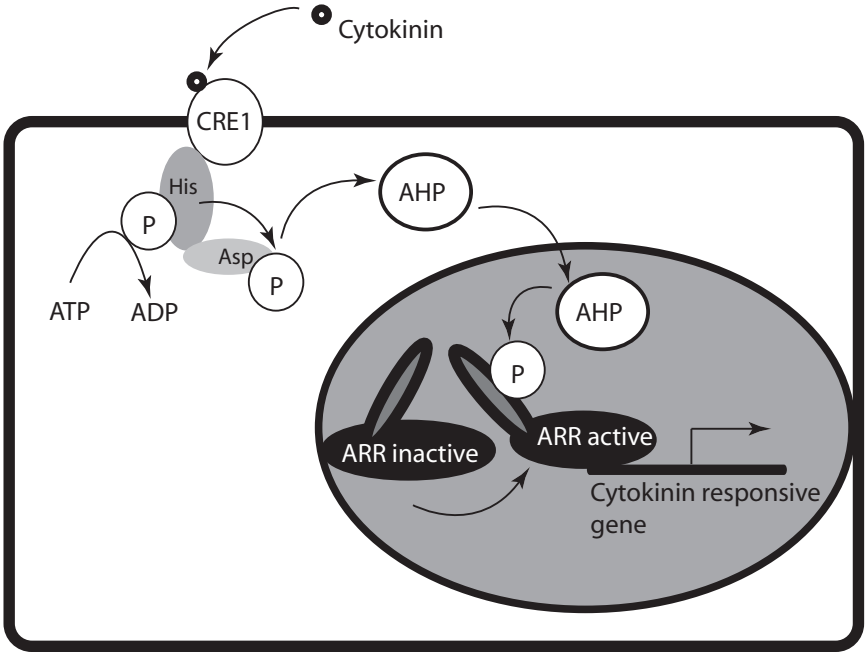

Figure 4. Cytokinin signal transduction (adapted from Aoyama & Oka, 2003). The CRE1 (AHK 1) histidine kinases (also AHK2 and AHK3) perceive cytokinins and their conserved histidine residues are phosphorylated. The phosphate is transferred to the Arabidopsis histidine-containing phosphotransfer factor, AHP, via the C-terminal receiver domain of the histidine kinase. The AHP carries the phosphate group into the nucleus and transfers it to an ARR (Arabidopsis response regulator), which transactivates cytokinin-responsive genes and an effect is mediated.

can bind to DNA and affect transcription. As illustrated in figure 4, a histidine kinase can sense an extra-cellular signal such as Zeatin, causing a histidine residue on the cytoplasmic side to become phosphorylated. This phosphate will then be relayed to an asparagine (asp) residue and then further to an *Arabidopsis* histidine-containing phosphotransfer factor (AHP). The AHP in turn will diffuse through the nuclear membrane and transfer the phosphate group to an *Arabidopsis* response regulator (ARR) which, in turn, will affect transcription of DNA by a cytokinin response gene (Fig. 4).

Yamada and co-workers demonstrated *in vitro* that cytokinins physically interact with the *CRE1* gene product and mediate a signal through the membrane (Yamada *et al*., 2001). Moreover, it has been demonstrated that AHP physically interacts with ARR1 (Suzuki *et al*., 2001b), and that the AHP interacts functionally with *CRE1* (Suzuki *et al*., 2001a). Sakai demonstrated that ARR1 is, indeed, a signal transducer that directly activates a primary cytokinin response gene (Sakai *et al*., 2001). *In situ*-hybridization revealed that vascular cylinder and pericycle cells specifically express *CRE1* in primary roots in Arabidopsis (Mähönen *et al*., 2000), therefore, it is likely that cytokinin signalling is involved in proliferation of root vascular precursor cells. Reverse transcriptase PCR analysis showed that expression of *CRE1* as well as *AHK2* and *AHK3* (two other cytokinin receptors that have been identified) overlaps

in roots, leaves, stems and flowers (Ueguchi *et al*., 2001b; Nishimura *et al*., 2004). Nishimura also demonstrated genetic redundancy for these genes when studying single, double and triple mutants of the *CRE1*, *AHK2* and *AHK3* (Nishimura *et al*., 2004). The *Arabidopsis* response regulators (ARRs) have been classified as type-A and type-B ARRs. Type-A ARRs are rapidly induced in response to exogenous cytokinins and this induction occurs in the absence of *de novo* protein synthesis (D`agostino *et al*., 2000). In contrast, the type-B ARRs have been shown to be the transcription factors that positively mediate cytokinin responses (Hwang & Sheen, 2001; Sakai *et al*., 2001). To and co-workers demonstrated with single to hextuple mutants in type-A ARR:s a progressively increasing sensitivity towards cytokinins, indicating that the type-A ARRs are negative regulators of type-B ARRs, possibly mediated by competition for phosphate groups from the AHPs (To *et al*., 2004). Cytokinin perception and signal transduction has been thoroughly reviewed by Kakimoto (2003) and Aoyama & Oka (2003).

Cytokinin Action

Cytokinins are believed to be involved in many processes throughout the plant life cycle. These include seed dormancy and germination, *de novo* bud formation, release of buds from apical dominance, leaf expansion, reproductive development and delay of senescence (reviewed by Mok, 1994). Furthermore, cytokinins are thought to be involved in blue light responses (Karnachuk, *et al*., 2001), red light perception (Fankhauser, 2002), regulation of source-sink relations (Roitsch & Ehness, 2000), water stress tolerance (Zhang *et al*., 2000), apoptotic induction (Carimi *et al*., 2003) and floral transition (Corbesier *et al*., 2003).

A central element of the function of cytokinins seems to be the ability to induce cell division via involvement in the cell cycle stages. Cell division proceeds via a continuous cycle of phases. Cells in the G1-phase expand and prepare for DNA replication which takes place during S-phase. After S-phase cells enter the G2-phase where they continue to expand and prepare for mitosis (cell division) which occurs during M-phase. In plant cells this process can be arrested at the G1 to S phase transition or at the G2 to M transition (den Boer & Murray, 2000). When cells are kept in the cycle, they keep on dividing and thus are prevented from undergoing further differentiation and assignation to a certain cell fate. This feature is utilized when growing callus (undifferentiated cells culture). Callus formation and growth require both cytokinins as well as auxins to be present. Reports of cytokinin increasing the amount of G1 Cyclin D3 (CycD3) required for the G1-S transition (Soni *et al*., 1995) and the demonstration that *Arabidopsis* callus constitutively expressing CycD3 can grow independently from cytokinin (Riou-Khamlichi *et al*., 1999) suggests that cytokinin is involved in the G1-S transition and thus keep cells dividing. Using synchronized tobacco cells, Redig demonstrated peaks of endogenous Z content around the S and M phases (Redig *et al*., 1996b). The potential role of cytokinin as a requirement in the G2-M phase transition was demonstrated by the finding that applications of lovastatin, an inhibitor of the mevalonate pathway (and therefore potential inhibitor of cytokinin biosynthesis to some extent) inhibited mitosis. Application of Z to these cells released them and they proceeded with mitosis (Laureys *et al*., 1998). The regulation of the cell cycle

is very complex and not completely understood. In plants more compounds, such as auxin, ABA, jasmonic acid and gibberelins appear to be involved (reviewed by Horvath *et al*., 2003).

By constitutively over-expressing cytokinin oxidase/dehydrogenase genes, Werner and co-workers managed to reduce endogenous cytokinin content by 30% to 60% (Werner *et al*., 2003). The phenotype included a severe reduction in the growth of aerial parts and reductions in internode length, leaf size and size of the shoot apical meristem (SAM). The cited authors showed that these phenotypical traits were due to a reduced rate of cell division, i.e. cell size increased while cell number decreased (Werner *et al*., 2003). They also demonstrated an increase in total root mass. It was therefore proposed that cytokinins are positive regulators of cell division in the shoot apical meristem and negative regulators of cell division in the root apical meristem. Furthermore, the transgenic tobacco displayed less apical dominance and delayed senescence. These findings conflict with the general view that cytokinins are positive regulators of lateral buds (resulting in less apical dominance) and that they delay senescence. Their endogenous cytokinin quantifications were, however, based on whole plant measurements and might not therefore reflect local conditions *in planta*, which might be of the greatest importance in cytokinin-regulated development (Faiss *et al*., 1997; Böhner & Gatz, 2001).

Another approach to investigate the roles of cytokinin *in planta* was taken by Rashotte *et al*. (2003). They applied various types of cytokinins in different concentrations to *Arabidopsis* plants and monitored subsequent gene expression over a time course by global mRNA profiling. Genes encoding the type-A Arabidopsis response regulators (type-A ARRs) and cytokinin oxidase were amongst the earliest and most significant to show positive responses in transcription. However, they also noticed increased expression of a specific cytochrome P-450 and of genes involved in auxin responses. Therefore, it seems that many of the early responses of cytokinins mediate changes in genes modulating either the cytokinin response (ARRs) or genes that can potentially regulate the active cytokinin pool (cytokinin oxidase and perhaps an iP-type hydroxylating cytochrome P-450). A similar experiment was performed by Hoth and co-workers. They used a dexamethasone-inducible system to elevate endogenous cytokinin levels (Hoth *et al*., 2003). They also found an induction of type-A ARRs as well as a transient induction of AHK4. Interestingly, they also noted a transient induction of AtIPT3, indicating that cytokinins might mediate positive feedback of their own biosynthesis (Hoth *et al*., 2003)

Taken together, these findings indicate that cytokinins are involved in many developmental processes and can respond to external signals during the course of plant life. The involvement of cytokinins in the cell cycle appears to be of central importance to cytokinin responses.

Auxin

Auxins are a class of plant hormones that naturally occur in all plants. The structure of auxin was determined by Thimann & Koepli (1935), who revealed it to be an indole derivative, indole-3-acetic acid (IAA). As well as their involvement in gravitropic and phototropic responses, auxins have been found to be involved in

many developmental processes, such as embryo development, differentiation of leafs and vascular tissue, primary and lateral root development, apical dominance and fruit development. The physiologically active form is the free acid (Fig. 1), but IAA can also be found in different conjugated forms, including ester-types with the carboxyl group linked via oxygen to a sugar (for example glucose) and amide-types with the carboxyl group forming an amide (peptide bond) to amino acids or polypeptides (thoroughly examined in Kowalczyk, 2002).

There appears to be a high level of redundancy in biosynthetic pathways leading to IAA. Two main routes are believed to exist (reviewed in Ljung *et al*., 2002). In one of the pathways tryptophan is the precursor. Ti plasmids of *Agrobacterium tumefaciens* T-DNA contain two IAA biosynthetic genes, iaaM and iaaH. Tryptophan is converted to indole-3-acetamide by tryptophan 2-monooxygenase (the gene product of iaaM) and then further hydrolysed to IAA by indole-3-acetamide hydrolase (the iaaH gene product). However, this is not believed to be the native biosynthetic route in plants. In endogenous biosynthesis, three intermediates in three separate pathways are candidates for the conversion of tryptophan to IAA. The intermediates are indole-3-pyruvic acid, tryptamine and indole-3-acetonitrile. In the tryptophan-independent pathway, indole-3-glycerol phosphate (IGP) is the putative precursor. In *Arabidopsis*, the greatest capacity to synthesise IAA *de novo* has been found in very young leafs, less than 0.5 mm in length, but all parts of the young *Arabidopsis* plant possessed the capacity to synthesise IAA *de novo* as demonstrated elegantly by Ljung and colleagues (Ljung *et al*., 2001).

The only good candidate identified so far for an auxin receptor is the auxin binding protein 1 (ABP1) (Timpte, 2001). One very important aspect of auxin responses is the modulation of gene expression. The most extensively studied subgroup of responsive genes consists of the early response genes, the induction of which occurs within minutes of exposure to elevated IAA levels and does not require protein synthesis. The *Aux/IAA* genes are included in this family of genes. In the current model for auxin-regulated gene expression Aux/IAA repressor proteins are associated with an Auxin response factor (ARF). This complex inhibits the expression of early auxin response genes. Auxin promotes the ubiquitination of Aux/IAA proteins, leading to their rapid degradation by the proteasome (Reed, 2001). In the absence of Aux/IAA repressor proteins, early response genes will become activated by ARF transcription factors and the auxin response is mediated by expression of early auxin response genes. With the use of Luciferase (LUC) fusion proteins Zenser and co-workers recently tested several plant hormones for their effect on IAA signal transduction. They demonstrated that only auxin can accelerate Aux/IAA proteolysis (Zenser *et al*., 2003). Two auxin resistant (auxin insensitive) mutants have been identified: *axr1* (Estelle & Sommerville, 1987) and *axr4* (Hobbie & Estelle, 1995). The *AXR1*gene product is believed to positively modulate ubiquitination, so the *axr1* mutant is insensitive to auxin when the degradation of Aux/IAA does not take place (Gray & Estelle, 2000). *Axr4* is probably not auxin insensitive in the sense that it causes a defect in the plant's auxin response, but is rather involved in the influx process of auxin into the cell (Yamamoto & Yamamoto, 1999). This was illustrated by the addition of NAA, which recovered the wild type phenotype, NAA as opposed to IAA possesses the ability to penetrate cells by means of diffusion rather than active uptake (Yamamoto & Yamamoto, 1999).

Cytokinin – Auxin Interactions

Cytokinins and auxins have been shown to interact in many different fashions, antagonistically, synergistically and additively (reviewed by Coenen & Lomax, 1997). Skoog and Miller demonstrated the importance of the kinetin/IAA ratio in organ formation from callus (Skoog & Miller 1957). The discovery by Wickson and Thimann (1958) that kinetin released outgrowth of buds in pea has been followed by more detailed studies of these interactions. One possible mechanism for the observed antagonistic behaviour would be reciprocal regulation of cytokinin and IAA, i.e. that the two groups of hormones control each other's biosynthesis and/or metabolism. Insertion of T-DNA from *Agrobacterium tumefaciens* has yielded crown gall tumours with increased cytokinin/IAA ratios (Akiyoshi *et al*., 1983; Smigocki & Owens, 1989). Bangerth observed an increase in cytokinin contents of xylem sap following decapitation in *Phaseolus* (Bangerth, 1994). A similar increase was observed in the node and internode below a point of decapitation in pea (Li *et al*., 1995) that preceded the outgrowth of the lateral buds. Expression of the bacterial *IPT* gene in tobacco has resulted in increased cytokinin levels and subsequent application of IAA has reduced the expression level of this gene as well as cytokinin levels (Zhang X. D. *et al*., 1996). Transgenic tobacco harbouring the bacterial *IPT* gene under the control of an auxin-inducible promoter, SAUR, showed increased tolerance to exogenously applied auxin in studies by Li et al. (1994). Unfortunately, no IAA quantifications were performed, but this could indicate lowered IAA levels. Eklöf and colleagues demonstrated that auxin over-producing plants displayed lower levels of cytokinins and lower cytokinin oxidase activity (Eklöf *et al*., 1997). Kotov and Kotova investigated hormonal changes in decapitated pea seedlings. Decapitation of 7-day old pea seedlings resulted in a 2-fold decrease in IAA levels in the 1st and 2nd internode, while the Z/ZR contents were increased 5-fold (Kotov & Kotova, 2000) Recently, an experiment with apically decapitated pea demonstrated a decrease in IAA in the stem followed by 5-6 fold increases in Z-type cytokinins and 1.5-2 fold increases in iP-type cytokinins in the stem (Kotova *et al*., 2004). In the roots, levels of iP-type cytokinins remained unchanged whereas contents of Z-type cytokinins increased 1.5-2 fold. In the root IAA levels remained constant compared to the control (Kotova *et al*., 2004).

These experiments give the impression that more IAA results in less cytokinins and less IAA results in more cytokinins. How this effect of IAA on cytokinin metabolism is mediated has not been completely clarified. However, some possible mechanisms have been proposed. The IAA conjugate IAA-glucose was found to inhibit the β-glucosidases that convert ZOG to the free base form, Z, thus potentially lowering levels of active cytokinins (Brzobohaty *et al*., 1994). Furthermore, auxin has been proposed to induce activity of cytokinin oxidase. This was demonstrated in tobacco pith explants in which oxidative breakdown of radio-labelled ZR was increased after the addition of NAA (Palni *et al*., 1988). Additional evidence was provided by the *in vitro* observation that the Z to adenine conversion rate increased after NAA treatment (Zhang *et al*., 1995). However, Motyka *et al.* (1992) did not detect any increases in cytokinin oxidase activity after applying several synthetic auxins to the surface of tobacco calli. Eklöf and co-workers detected a reduction

in cytokinin oxidase/dehydrogenase acitivity in IAA overproducing tobacco plants (Eklöf *et al*., 1997).

Cytokinin effects on auxin levels have also been investigated. Expression of the bacterial *IPT* gene from Agrobacterium can cause auxin autonomy and increase the IAA content in *Nicotiana glutinosa* cells (Binns et al., 1987), while *Zea mays* roots immersed in media containing Z have displayed elevated IAA levels (Bourquin & Pilet, 1990). Increases in IAA levels have been observed following applications of BAP (benzyl amino purine) to pea (*Pisum sativum*) root tips (Bertell & Eliasson, 1992) and application of exogenous synthetic cytokinins to pea apices resulted in increased levels of free and conjugated IAA in the second node and internode in a study by Li & Bangerth (2003).

In contrast to these findings, using a bacterial *IPT* over-expressing tobacco system Eklöf and co-workers demonstrated a reduction in IAA pool sizes and biosynthesis at the whole plant level (Eklöf *et al*., 1997). Furthermore, perceptions about the importance of the cytokinin/auxin ratio became somewhat more complex when plants over-expressing both IAA and cytokinin biosynthetic genes were examined. Whole plant extracts of these plants had wild type levels of both hormones. However, they simultaneously displayed both auxin and cytokinin over-producing phenotypes (Eklöf *et al*., 2000). Furthermore, in an *Arabidopsis* mutant, SUPERSHOOT, which is defective in the *sps* gene encoding a cytochrome P-450, zeatin levels were elevated 3-9 fold compared to wild type (Tantikanjana et al., 2001). Simultaneously, the IAA level was eight times higher than wild type (Tantikanjana et al., 2001).

How cytokinins regulate auxin metabolism has also not been clarified. However, cytokinins inhibited the conjugation of IAA to IAA-aspartate in mungbean hypocotyls in experiments by Yip & Yang (1986), thereby possibly increasing the levels of free IAA.

The synergistic effect of cytokinins and auxins in the cell cycle has also been demonstrated. It was early shown that kinetin alone does not induce mitosis (Das *et al*., 1956). However, addition of IAA and kinetin to cultured tobacco pith tissue induced mitosis (Das *et al*., 1956). Regulation of the G2-M transition is probably mediated by activation of Cyclin Dependent Kinases (CDKs). In tobacco pith explants, application of auxin increased the immuno-detectable amount of CDK protein and additional application of cytokinin resulted in activation of CDK, possibly through de-phosphorylation of CDK (Zhang K. *et al*., 1996). Auxin induces expression of the cyc2 class of CDKs, and cytokinin increased the catalytic activity of its gene products when examined in shoot-derived cell suspensions of tobacco by John *et al*. (1993). Another CDK transcript, CycD3, was found to be induced by cytokinins in a cytokinin over-producing mutant (Nogue *et al*., 2000). Furthermore, cell division can be induced and maintained in the absence of exogenous cytokinins in transgenic plants over-expressing CycD3 (Riou-Khamlichi *et al*., 1999). Therefore, cytokinins and auxin appear to co-regulate shoot cell proliferation by controlling the activation and expression of cell cycle components. A gene (*PROPORZ1*) possibly involved in the cytokinin and auxin mediated signalling during the shift from cell proliferation to differentiation was recently identified (Sieberer *et al*., 2003). When the mutant was grown in callus tissue, it lacked the ability to form organs in response to altered cytokinin/auxin ratios.

Experimental

Plant material and growth conditions

Paper **I** describes studies with *Arabidopsis thaliana* ecotype Landsbergis erecta and a line (3-2) transformed with the *Agrobacterium tumefaciens IPT* gene. In the 3-2 line the *IPT* gene was under the control of a glucocorticoid-inducible expression system which was induced by the addition of dexamethasone to the growth media (Aoyama & Chua, 1997; Kunkel *et al*., 1999). The use of an inducible system circumvents problems associated with constitutive over-production of a hormone and the potential alterations in growth compared with wild type control. For the study in paper **II**, *Arabidopsis thaliana* ecotype Columbia was used, as well as the inducible (3-2) line expressing the *Agrobacterium tumefaciens IPT* gene. In paper **II** *Nicotiana tabacum* L. cv. Petit Havana SR1 was also examined, as well as Arabidopsis mutants *axr1-3*, (Estelle & Sommerville, 1987) *axr4-2* (Hobbie & Estelle, 1995) and the Arabidopsis double mutant *axr4-2 x aux1-7* (Hobbie & Estelle, 1995). To mimic the effect of IAA we used NAA (1-naphthaleneacetic acid), which (unlike IAA) can enter cells by diffusion, thus ensuring a direct effect mediated simultaneously throughout the plant. For paper **III** *Arabidopsis thaliana* ecotype Columbia was used. Transgenic line *gt6-2* of *Arabidospsis thalina* ecotype Landsbergis erecta, and a double mutant *gt6-2* x *stm1* were used in paper **IV**.

In the studies described in Papers **I** and **II**, *Arabidopis* were grown sterile in liquid culture in 1/1-Murashige and Skoog (MS) media **(I)** and 1/2 MS media **(II)**. The media contained 1.5% sucrose (1/2 MS) and the pH was adjusted to 5.6 using KOH. Approximately 25 seeds were added to each 250 ml Erlenmeyer flask which contained 50 ml media. Seedlings were grown for three weeks at 22°C under long day conditions (18h light/6h dark). In **II** tobacco was grown for 6 weeks on soil before incubation. In **III** Arabidopsis was grown on soil under short day conditions (9h light) for three months.

Sample purification

In these studies we employed two different extraction and purification strategies. In **I** and **II**, Bielesky buffer extraction (60% methanol, 25% chloroform, 10% formic acid and 5% water) was used followed by purification through SCX (a cation exchanger), DEAE (an anion exchanger), C18 (reversed phase) solid phase extraction (SPE) cartridges, and finally immunoaffinity chromatography (IAC) as described in Åstot *et al*. (1998). In **III** and **IV**, a new strategy was developed which was based on cytokinin purification as described by Dobrev & Kaminek (2002). This approach used an acidified water-methanol mixture for extraction followed by C18 and MCX (Mixed Mode; Cation-Reversed Phase, purification cartridge). A schematic presentation of the setup is displayed in figure 5. No immunoaffinity step was utilised, and a certain compromise on purity was observed. However, this strategy proved to be sufficiently robust, moreover it proved to be easy to automate using a positive-pressure SPE-robot.

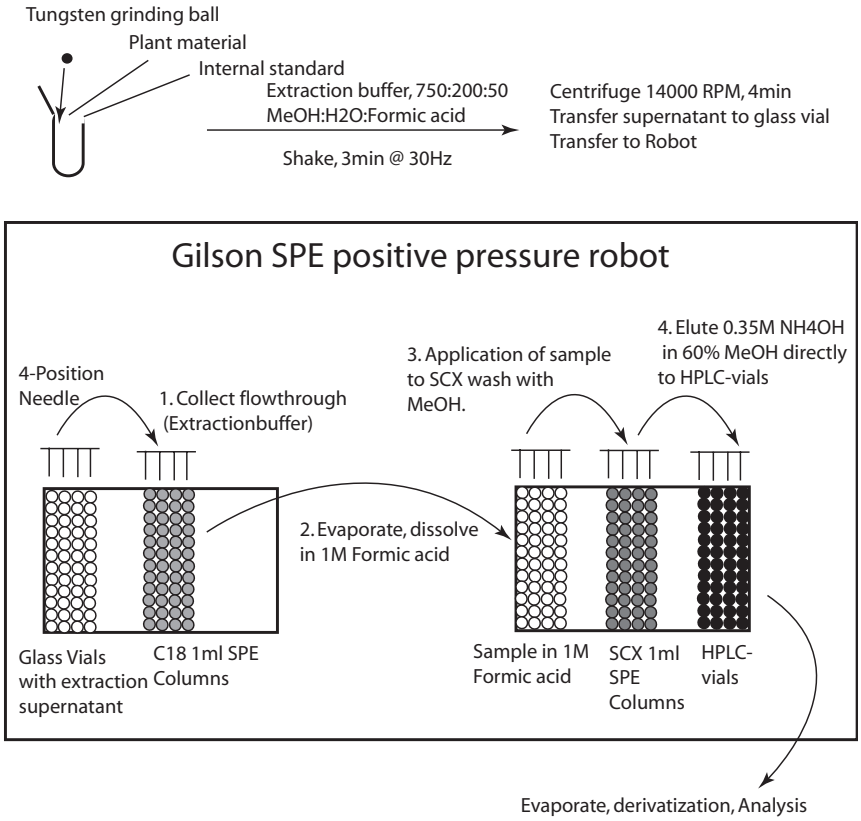

Figure 5. Extraction and purification work flow (**III**)

Electrospray Mass Spectrometry (ESI-MS)

Cytokinin quantifications described in Paper **I** were performed with capillary liquid chromatographic/frit-fast atom bombardment. The use of liquid chromatography coupled with fast atom bombardment (FAB) for cytokinin analysis is described in detail in Åstot *et al*. (1998). In **II**, **III** and **IV** liquid chromatography hyphenated via electrospray interface (ESI) to a triple quadrupole mass spectrometer was used for cytokinin separation and detection. In Liquid Chromatography-Electro Spray Interfacing/Ionisation - Mass Spectrometry (LC-ESI-MS), the LC effluent is transported through a capillary to which a high voltage (2-5 kV) is applied. This voltage can be either positive or negative, depending on the analytes. The applied voltage provides the electric field gradient that gives rise to charge separation on the surface of the liquid. A "Taylor cone" is generated, which protrudes from the capillary tip (Fig. 6). When the solution creating the Taylor cone reaches a point at which the Columbic repulsion of the surface charge is equal to the surface tension of the solution, the Rayleigh limit (Taflin *et al*., 1989), droplets that contain excess positive or negative charge detach from the tip. These droplets move through the

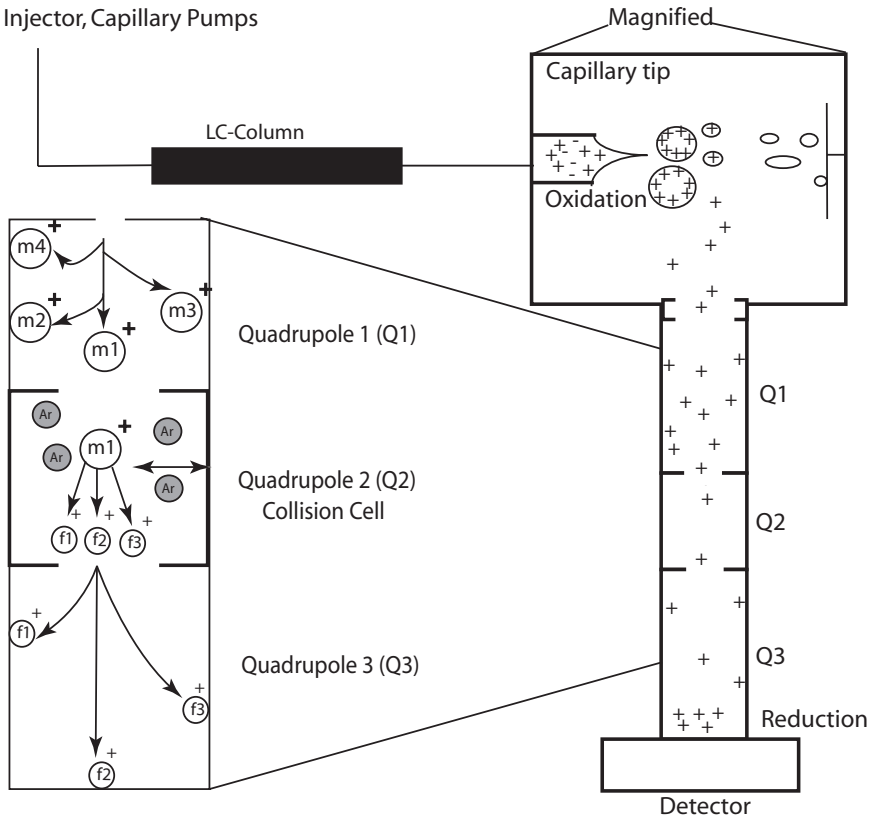

Figure 6. Schematic view of Electro Spray Ionization (ESI). Liquid is transferred from the separation column through a stainless steel capillary to which a voltage is applied. At the tip of the capillary, a "Taylor cone" is formed. Droplets are released from the cone when the Rayleigh limit is reached. Eventually this leads to formation of free ions. Uncharged molecules, such as solvent, are not extracted with a setup like this (orthogonal ion extraction). In Multiple Reaction Monitoring, parent ions are selected in the first quadrupole. Only m1 passes Q1 with a stable trajectory. In Q2 (the collision cell), fragmentation is induced with argon gas and an energy field. Only a certain daughter fragment (f2) passes Q3 with a stable trajectory and is detected at the detector. Only an electronic signal is generated in this mode, and no spectral information is obtained in MRM analysis.

atmosphere towards the entrance of the mass spectrometer and generate ions by a mechanism that is still to be fully explained (Kebarle & Peschke, 2000). Charging of the new surface as the droplets form and leave the tip requires a continuous flow of charge from the power supply and the electrospray process can be described as one in which oxidation takes place at the spray tip and reduction on the counter metal plate (Fig. 6) in positive ESI (in negative ESI oxidation/reduction is reversed).

Ionization takes place under atmospheric pressure in ESI, in contrast to FAB, where the molecules are continuously introduced to the ion source which is held at low pressure. The utilization of atmospheric ionisation puts less constraints on the flow rates which can be used for the separation system i.e. higher flow rates can be

used. After the charged molecules have been produced in the ESI process, they enter the first quadrupole. This is a low pressure region, evacuated by rotary pumps and turbo pumps to the region of 10^{-6} torr. The quadrupole consist of two pairs of rods arranged orthogonally to each other. Each pair of rods employs a combination of direct-current (DC) and radiofrequency (RF) fields as a mass "filter". The "filter" is scanned by ramping the magnitude of the RF amplitude and DC voltages at a fixed ratio. The RF frequency is held constant. For any given set of RF and DC voltages only ions of a specific m/z will obtain a stable trajectory and reach the detector. The mass spectrum is scanned as the voltages are swept from a pre-established minimum to a maximum value at a constant DC:RF ratio. In **II**, **III** and **IV**, we utilized a triple quadrupole which has three quadrupoles aligned in series. The middle one is operated solely with radio frequency and will not select ions of a particular m/z, but is rather used as a collision cell. The triple quadrupole can perform many different ms/ms operations. We have utilised it for Multiple Reaction Monitoring (MRM), which is the optimal mode for targeted analysis. The first quadrupole is set to pass only a specific mother fragment, in our case the Molecular ion + Proton ($M+H^+$). The chosen ion is then induced to fragment in the collision cell with the aid of energy and a gas (Argon), and a specific fragment is selected in the third quadrupole (Fig. 6). The result is highly specific monitoring of a certain parent ion to daughter ion m/z transition. The quadrupoles are swept constantly between certain transitions and the output is a measured current in the detector, which can be integrated and a signal proportional to the amount of analyte is obtained.

Plant hormone quantification

In order to compensate for possible losses during sample clean up or derivatization and variations in the amounts injected onto the columns, we used internal standards. These were cytokinins labelled with five to six stable-isotope deuterium (^2H) atoms. The nucleotides were also labelled, with one ^{15}N-atom. Quantifications were based on calculated response ratios in the samples between the endogenous, unlabelled, cytokinins and their labelled counterparts. These response factors were then related to a pre-constructed calibration curve with different ratios between the endogens and the internal standards. Using this procedure, an absolute value for the endogenous concentration present in the sample can be obtained. A summary of standards used is displayed in table 1.

In Vivo deuterium labelling experiments

In **I, II** and **IV** *in vivo* deuterium labelling experiments were performed to trace *de novo* cytokinin biosynthesis. Plants were grown to a certain stage on normal MS media than transferred to media that contained 30% ^2H_2O. Heavy water is a tracer that avoids certain problems associated with stable isotope labelled precursors, such as compartmentalization in a non-native fashion, as illustrated with $^2H_5$-Trypthophan when tracing IAA biosynthesis (Rapparini *et al*., 1999). ^2H_2O will have access to all compartments of the cell and the introduction of this tracer will start a general labelling process in all metabolic pathways of the plant. An LC-(frit)-FAB method for measuring *in vivo* deuterium labelling *in planta* was developed by Åstot

Endogen	Internal Standard	Endogen m/z-transition	Internal Standard m/z-transition
iP	$^{2}H_{6}$-iP	204-136	210-137
Z	$^{2}H_{5}$-Z	276-202	281-207
DHZ	$^{2}H_{5}$-Z	278-204	281-207
iPA	$^{2}H_{6}$-iPA	504-204	510-210
ZR	$^{2}H_{5}$-ZR	576-276	581-281
DHZR	$^{2}H_{5}$-ZR	578-278	581-281
iPMP	$^{2}H_{6}$,$^{15}N_{1}$-iPMP	528-204	535-211
ZMP	$^{2}H_{5}$,$^{15}N_{1}$-ZMP	600-276	606-282
ZOG	$^{2}H_{5}$-ZOG	606-202	611-207
ZROG	$^{2}H_{5}$-ZROG	906-606	911-611
Z7G	$^{2}H_{5}$-Z7G	662-276	667-281
Z9G	$^{2}H_{5}$-Z9G	662-276	667-281

Table 1. Standards and m/z transitions used in the work.

and co-workers (Åstot *et al*., 2000b). The plants rapidly incorporated deuterium into the side chain and ribose moiety. In the adenine moiety of cytokinins not much label was found, demonstrating recycling of this structure by the plants. In order to measure the biosynthetic rate of cytokinins specifically, the MRM transition from a fragment that did not contain the ribose moiety was required. For Z type cytokinins the m/z transitions used in **I**, **II** and **IV** were: 276-202, 277-203, 278-204 and 279-205. These transitions reflected the loss of the propionyl group from the "base and side chain-propionyl group" fragment. In **I** single ion monitoring (m/z 204, 205, 206 and 207) in the high resolution mode was utilised for studying the incorporation into iP-type cytokinins. In **II** and **IV** MRM was also used for the iP-type using an ESI-triple quadrupole configuration. This was possible since the loss of the side chain fragments at m/z 69, 70 and 71 was observed from the corresponding "base-side chain" fragments at m/z 204, 205 and 206. The incorporation was then calculated and presented as a tracer/tracee ratio (t/t-ratio). The t/t-ratio is obtained by calculating the natural isotope distribution in a set of standards, then subtracting the natural isotope contribution for each isotopomer in the sample by multiplying the natural isotope fraction (%) found in the standards by the I0 isotope ($M+H^{+}$) and subtracting the resulting value from the respective isotopomers (I1, I2 etc.). This is followed by dividing the summed natural isotope corrected-intensities in I1, I2 etc by the sum of the natural isotopes (Fig. 7).

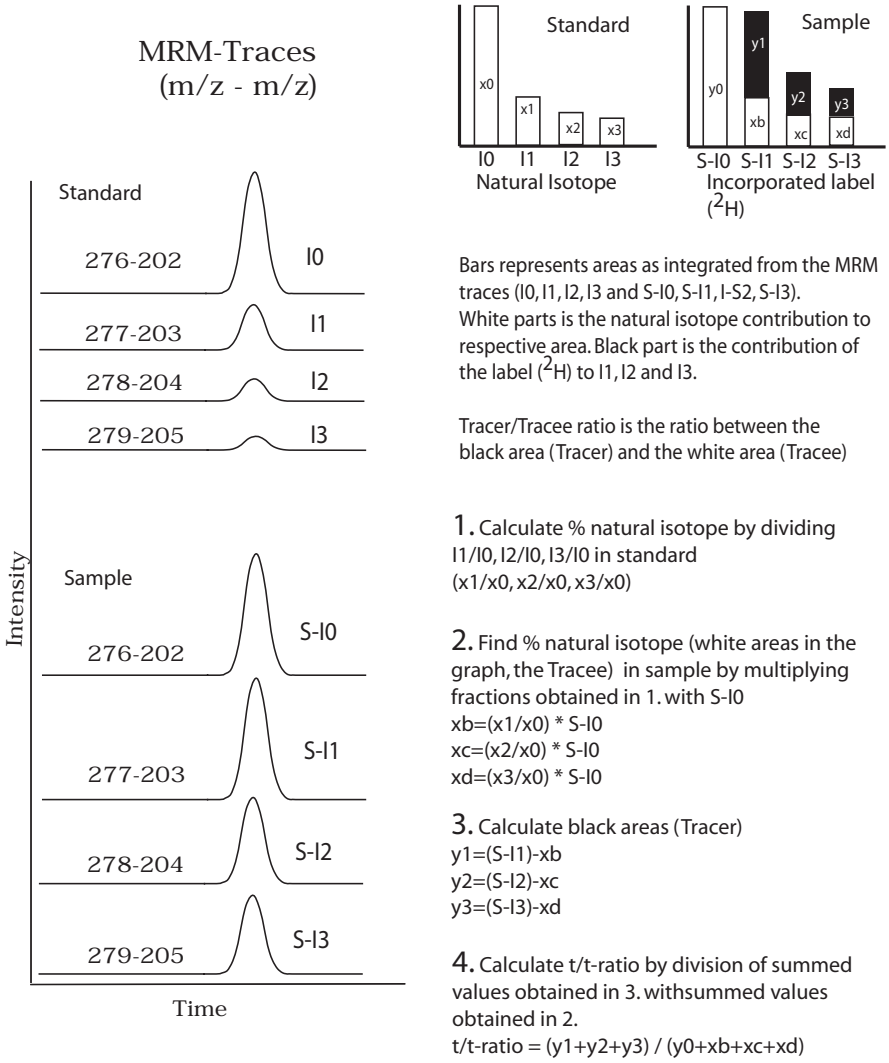

Figure 7. Schematic presentation of calculation of t/t-ratios (*de novo* synthesis)

Results and Discussion

De novo ZMP synthesis (I)

The primary event of cytokinin biosynthesis has generally been believed to be the formation of iPMP by the attachment of DMAPP to AMP via the action of an isopentenyltransferase enzyme (Fig. 2) (Chen, 1997), which was identified in *Agrobacterium tumefaciens* as IPT (Barry *et al*., 1984; Akiyoshi *et al*., 1984). In **I** we used an inducible transgenic *Arabidopsis* line that expresses the bacterial IPT after induction with the glucocorticoid dexamethasone (Kunkel *et al*., 1999). It was found that ZMP levels increased 100-fold within 12 h of induction whereas over-

expression of the bacterial *IPT* caused not even a transient increase in the level of iPMP (**I**). This suggested either that ZMP was synthesised *de novo* or that iPMP to ZMP conversion remained very rapid even with a dramatic increase in iPMP biosynthesis. Further experiments in which we measured *de novo* biosynthesis of ZMP and iPMP in the inducible *Arabidopsis IPT* line with *in vivo* deuterium labelling revealed that the iPMP biosynthetic rate was close to linearly constant during a 24h experiment (**I**). For ZMP, on the other hand, there was a distinct increase in the biosynthetic rate shortly after induction with dexamethasone. After 24 hours, the t/t-ratios were 2.5 and 3.5 for iPMP and ZMP, respectively (**I**). This is not consistent with an iPMP biosynthetic pathway. In a linear biosynthetic pathway a precursor cannot have a lower level of enrichment than its product. In a further attempt to explain the data a double tracer experiment was performed, as illustrated in figure 8. Feeding plants with simultaneously with $^{2}H_{6}$-iPA and $^{2}H_{2}O$ would result, if most ZMP is formed via iPMP, in a distinct increase in the +5 isotopomer of ZMP due to the five deuterium atoms that follow the iPA side-chain via hydroxylation of iPMP in the conversion to ZMP (Fig. 8). Simultaneous feeding of 30% $^{2}H_{2}O$ and $^{2}H_{6}$-iPA to transgenic *Arabidopsis* induced to over-express the bacterial IPT gene resulted in an isotopomer pattern typical of *de novo* synthesis (**I**) and no distinct signal in the +5 channel of ZMP. From this we can conclude that in the inducible IPT system ZMP is synthesised *de novo* independently of iPMP. Also, when we blocked hydroxylation of iPMP to ZMP with metyrapone (Fig. 8) we observed *de novo* ZMP synthesis (**I**). But what is the case for wild type (wt) *Arabidopsis*? In a similar double tracer feeding experiment using wild type plants, it was demonstrated that some ZMP formed originated from hydroxylated iPMP (giving a distinct signal for the +5 isotopomer for ZMP) (**I**). But there was also *de novo* synthesis when hydroxylation was blocked (Fig 8). This demonstrated that wild type *Arabidopsis* possesses the capacity to synthesise ZMP *de novo* (**I**). An experiment with mevastatin, an inhibitor of the cytosolic mevalonate pathway and metyrapone, a P-450 inhibitor, inhibiting hydroxylation, resulted in significantly reduced ZMP *de novo* biosynthesis (**I**), indicating that the side chain precursor is synthesised in the cytosol. However, it should be noted that this is not entirely consistent with recent findings (see below).

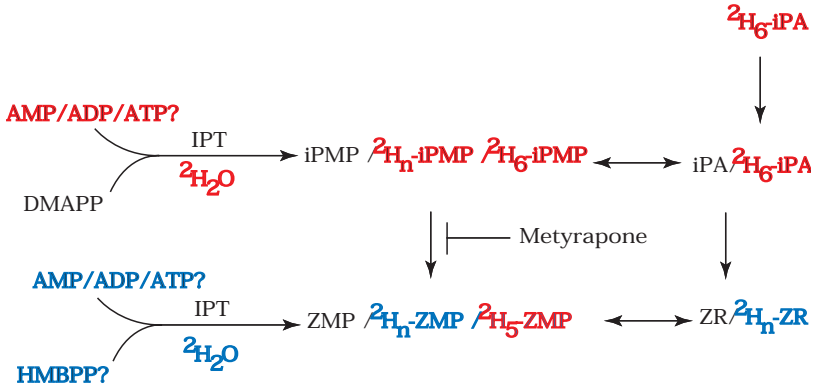

Figure 8. Double tracer feeding experiment. Plants are incubated with both $^{2}H_{6}$-iPA and $^{2}H_{2}O$. The labelling pattern reveals *de novo* biosynthesis of ZMP if there is no distinct +5 isotopomer for ZMP when hydroxylation is blocked with metyrapone, and labelling is distributed instead amongst the I1 to I5 isotopomers.

The putative side chain precursor would be HMBPP, this molecule has been identified as an intermediate in the non-mevalonic, and plastid-localized, DXP-pathway of *E. coli* (Hecht *et al*., 2001) (Fig. 2). In our experiment we utilized mevastatin, which is known to selectively inhibit HMGA coA reducatase in the mevalonic pathway, which is localised in the cytosol (Endo *et al*., 1976). It has recently been demonstrated that trans-cytokinins in *Arabidopsis* are of plastid origin, whereas cis-cytokinins seem to be of cytosolic origin (Kasahara *et al*., 2004). Furthermore, a loss of function mutant in *Arabidospsis hmg1*, which has a malfunction in expression of the HMG coA reductase of the mevalonic pathway, were not affected in cytokinin levels (Suzuki *et al*., 2004), indicating that cytokinins are synthesised from side chains that are of non-cytosolic origin. There has been speculation concerning the possible transfer of IPP between the plastid and cytosol (Lichtenthaler, 1999). If IPP could diffuse between these two compartments, IPP could be transformed to DMAPP in the cytosol and thus act as a side chain precursor to AMP, subsequently yielding cytokinins even in the presence of mevastatin. However, we also added metyrapone, thereby blocking the possibility of ZMP arising from iPMP. Furthermore, recent studies using virus-induced gene silencing in tobacco have ruled out the possibility of significant transfer of IPP between the plastid and the cytosol (Page *et al*., 2004). The reduction in *de novo* ZMP biosynthesis we observed when adding mevastatin is difficult to explain in the light of these findings. However, the finding of *de novo* iPMP-independent ZMP biosynthesis still appears to be valid, and is further supported by observations of Krall *et al*. (2002) who recorded ZMP formation *in vitro* following applications of radiolabelled HMBPP and AMP using the *Agrobacterium TZS* enzyme. Also, Takei observed production of Z-type di- and tri-phosphates using AtIPT7 enzyme obtained from heterologous expression in *E. coli*, with HMBPP as substrate (Takei *et al*., 2003).

Reciprocal Regulation of Auxin – Cytokinin Pool Sizes and *de novo* Biosynthesis (II)

In Paper **II** we explored the metabolic basis of observed reciprocal cytokinin-auxin effects on each other's metabolism. Wild type *Arabidopsis* plants were grown in liquid culture as described in the *Experimental* section. The IAA analogue 1-napthaleneacetic acid (NAA) was applied and 24 h after incubation with NAA a reduction in the level of several cytokinins was observed (**II**). The use of NAA instead of IAA is preferred because NAA can rapidly access cell compartments and thus ensure fast effects, whereas IAA does not have free access to the cell through diffusion. The next step was to design a dose-time experiment in which NAA was applied at different concentrations and samples were collected in a time course. *De novo* synthesis was measured for ZMP, ZR and Z9G and the results are shown in (**II**). The negative effect of NAA on cytokinin biosynthesis was observed after just 6 h for ZMP and ZR. After 24 h a significant decrease in Z9G biosynthesis was observed. This is in agreement with the view that cytokinins are synthesised *de novo* at the nucleotide level (Fig. 3).

We then proceeded with a similar experiment where we used the inducible IPT system in order to monitor the effect of elevated cytokinin levels on IAA levels and biosynthesis (**II**). Using an inducible system has the advantage that there are no growth alterations as compared with wild type up until induction. Constitutive over-production of a hormone might induce changes prior to measurements and make it difficult to distinguish between primary and secondary effects. The very steep increases in ZMP and Z levels did not have an immediate effect on the IAA pool size or *de novo* synthesis. A reduction in IAA biosynthesis was observed between 24 and 36 h, and after 48 h a significant reduction in IAA compared with the control was observed. The conclusion was that IAA exercises very tight control of *de novo* cytokinin biosynthesis, whereas cytokinins seem to affect IAA biosynthesis indirectly through developmental changes rather than via a direct control mechanism (**II**). This can be contrasted to other findings suggesting a positive effect of cytokinins on IAA levels (Li & Bangerth, 2003). The cited authors measured an increase in the amount of IAA released below a point of treatment with synthetic cytokinins in pea. One must however remember that our conclusions are based on data obtained from whole plant measurements. What we can conclude with certainty is that the net effect of NAA on cytokinin metabolism is a reduction in biosynthesis and that cytokinins have similar effects on net IAA biosynthesis and pool size. Locally, auxin/cytokinin ratios might be very different, as observed by Eklöf *et al.* (2000). In tobacco plants over-expressing both the cytokinin IPT gene and IAA biosynthetic genes, wild type levels of both hormones were observed. At the same time the plant displayed phenotypical traits of both cytokinin and IAA over-production (Eklöf *et al*., 2000).

Mechanism of IAA regulation of Cytokinin Biosynthesis (II)

In order to investigate how NAA exercises its effects on cytokinin biosynthesis in wild type plants we designed an experiment where we decoupled the two *de novo* biosynthetic routs of cytokinins from each other by adding metyrapone to block the hydroxylation of iPMP to ZMP (Fig. 8), and 0/25 µM NAA. The results were striking (**II**). There was no difference in *de novo* iPMP biosynthesis following the addition of 0 or 25µM NAA, but there was a significant decrease in the biosynthesis of ZMP when NAA was added. This implies that NAA acts primarily on the iPMP-independent, *de novo* ZMP biosynthetic pathway. Furthermore, we examined the effects of adding NAA on *de novo* ZMP biosynthesis in an auxin-insensitive mutant (*axr1*) and an auxin mutant (*axr4*) which has altered IAA influx capability (Yamamoto & Yamamoto, 1999) **(II)**. Since *axr4* is basically restored with the addition of freely diffusible NAA whereas *axr1* is defective in auxin signal transduction, it was judged that this experiment would provide strong indications about the potential role of auxin signal transduction in IAA-regulated cytokinin biosynthesis. The auxin mutant *axr4* displayed a similar reduction in biosynthesis of ZMP as the wild type control, whereas NAA had less effect on ZMP biosynthesis in the auxin-insensitive mutant *axr1*. This indicates that normal auxin perception and signal transduction is needed for the auxin effect on cytokinin biosynthesis to be mediated (**II**).

Sites of Cytokinin Biosynthesis (II)

The current model of cytokinin-auxin regulated development is based on concentration ratios of these two hormones. Our results, showing a very rapid control of cytokinin biosynthesis by IAA impacts this model, especially since cytokinins have been found to exert their effects to some extent in a paracrine fashion (Faiss *et al*., 1997; Böhner & Gatz, 2001) rather than being a long distance signalling molecule. Furthermore, the plants' potential capacity for IAA synthesis in different sites was recently investigated by Ljung and co-workers. They found that young leaves, which contain tissues undergoing intense cell division, had the highest IAA biosynthetic capacity (Ljung *et al*., 2001). Moreover, they showed that all parts of the *Arabidopsis* plants had capacity for IAA synthesis. Therefore, we wished to investigate potential sites of cytokinin biosynthesis. Firstly we dissected *Arabidopsis* root and shoot tissues and incubated them separately in 30% $^{2}H_{2}O$. The resulting isotopomer patterns are displayed in (**II**). The istopomer profiles revealed *de novo* ZMP synthesis in both root and shoot tissues. Even though most evidence has indicated that cytokinins are synthesised in roots (reviewed by Letham, 1994), some experiments have indicated that cytokinin synthesis may occur in the shoot apex (Chen & Petschow, 1978). Expression studies of endogenous AtIPT cytokinin biosynthesis genes by Miyawaki and co-workers demonstrated that *AtIPT1* and *AtIPT7* were expressed in apical parts (Miyawaki *et al*., 2004) further strengthening the possibility of apical *de novo* synthesis of cytokinins.

In order to obtain a higher resolution understanding of the localisation of cytokinin biosynthesis in the apex we switched to *Nicotiana tabacum* (**II**). Leaves of six-week-old tobacco plants were dissected out according to their size. Large leaves (>20 cm length), medium-sized leaves (between 20 and 5 cm in length) and small leaves (<5 cm in length) were all incubated separately in $^{2}H_{2}O$. The highest biosynthetic capacities of ZMP and ZR were found in the smallest leafs, which also contained the largest pools of these metabolites (**II**). This should be related to the findings of Ljung, who found that small leaves possess the highest IAA biosynthesis capacity (Ljung *et al*., 2001). How does this relate to our finding that IAA rapidly regulates cytokinin biosynthesis? Ljung also found a concentration gradient of IAA in the leaves, with IAA concentrations increasing towards the petiole (Ljung *et al*., 2001). One possible explanation for these findings is that the auxin-cytokinin interaction and regulation may occur in highly localised "micro environments" in the developing plant. If so, whole plant experiments might give misleading results, since tobacco plants over-expressing both cytokinin and auxin biosynthetic genes display phenotypical traits of both cytokinin and auxin over-production, but have wild type levels of both hormones (Eklöf *et al*., 2000). In Paper **II** we further addressed questions concerning root and shoot localised biosynthesis by using the Arabidopsis double mutant *axr4-1* x *aux1-7*. This mutant has impaired lateral root formation, but neither *de novo* ZMP biosynthesis nor the ZMP pool was altered as compared to wild type. However, the iPMP-pool was strongly reduced, suggesting that different cytokinin metabolites may have different sites of synthesis **(II)**.

Derivatization of Cytokinins for ESI-MS/MS (III)

Cytokinin analysis poses a great challenge for the investigator. Cytokinins are generally present in very low amounts in plants: 0.05 – 50 pg / mg fresh weight. Plants also contain thousands of other compounds of widely differing sizes (proteins to small molecules), each with different physical characteristics. Therefore the cytokinins first have to be enriched by suitable means of sample purification, then separated from each other and detected. Cytokinins as a group are quite diverse, ranging from free bases (Z and iP) through ribosides and glucosides (ZR, iPA, ZOG) to polar nucleotides (ZMP and iPMP). However, they share some common physical features that can be used for analyte enrichment. All cytokinins are ionisable depending on pH. The exocyclic amino group at position 6 of the purine has a $pK_a \approx 4$ giving cytokinins a positive charge at pH<2. The imidazole hydrogen (NH) at position 9 has a $pK_a \approx 10$, giving rise at pH>12 to a negative charge (Horgan & Scott, 1987). The cytokinin nucleotides, in addition to their basic pK_a at ≈ 4, have acidic pK_a values at ≈ 1 and 6 associated with the phosphate group. Thus at pH ≈ 2 the nucleotides will behave as zwitterions with no net charge and as pH is increased above 6 they will become doubly negatively charged (Horgan & Scott, 1987). Furthermore, the hydrophobicity of cytokinins differs from that of analagous adenine structures because of the side chain structure attached at the N^6 position. Different strategies to enrich cytokinins have been employed (Prinsen *et al*., 1995; Åstot *et al*., 1998; Witters *et al*., 1999; Dobrev & Kaminek, 2002; Novak *et al*., 2003). All of these authors but Dobrev & Kaminek used immunoaffinity purification as the final step. This generates highly enriched samples since immunoaffinity-chromatography (IAC) offers retention factors (k) approaching infinity for their specific analytes, whereas the retention factors for all other compounds are theoretically zero, that is, only the analyte for which the columns have specific affinity are retained, and all other metabolites pass through them. However, this purification strategy is not easy to automate and the IAC-columns available today have no affinity for N7-glucosides and O-glucosides or intact nucleotides. Therefore, we developed a purification scheme **(III)** based on information in Dobrev & Kaminek (2002) (Fig. 5). We automated this purification procedure using a positive pressure SPE robot and obtained a throughput of 70 samples/day (routinely). This can be compared with 6-12 samples/day using previous methods (**III**).

In the studies described in Paper **I** we used a Liquid Chromatography – Fast Atom Bombardment setup (LC-FAB) for separating and detecting cytokinins. A derivatization scheme based on the propionylation of hydroxyl groups was developed by Åstot and co-workers for improved sensitivity (Åstot *et al*., 1998). In **III** we further developed the derivatization strategy and employed it for Electrospray Ionisation (ESI) mass spectrometry. The principles of ESI are outlined in figure 6. By coupling a propionyl or benzoyl group to available hydroxyl groups, a more hydrophobic cytokinin derivative is obtained (Fig. 9). This gives the molecules greater “surface activity”, which is a desirable feature during the ionization process in ESI. The more hydrophobic molecules tend to concentrate at the interface between the droplet surface and atmosphere. Thus, they are easier to ionize than more polar molecules, which more readily dissolve in the solvent droplet and, hence, are discriminated against when repulsion causes the droplets to shrink (Tang

& Kebarle, 1993; Cech & Enke, 2001a). The possibility of using derivatization for ESI was illustrated in work by Okamoto who achieved a 5000-fold increase in the ESI signal for maltopentaose by reacting it with trimethyl (p-aminophenyl)-ammonium (Okamoto *et al*., 1995). The effect of derivatization on ESI signal intensity is illustrated for some cytokinin derivatives and ADP in (**III**).

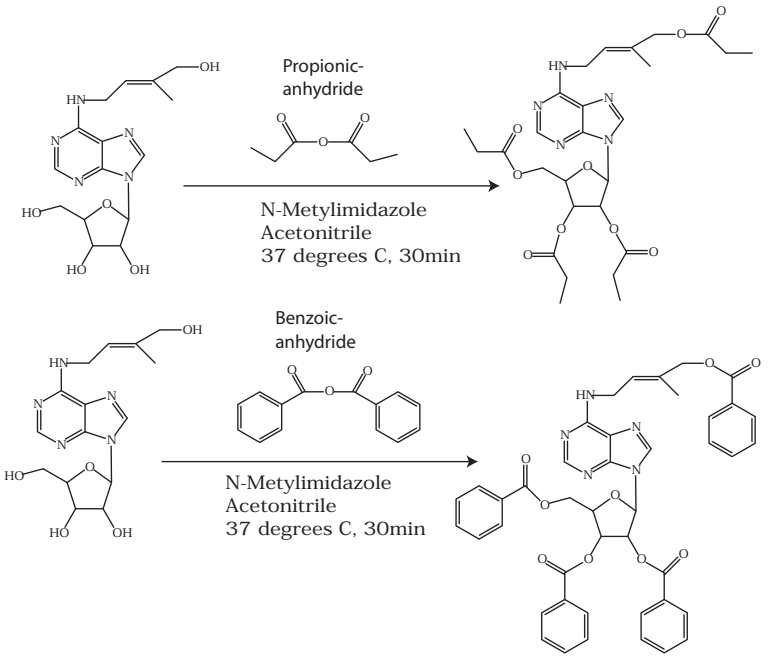

Figure 9. Derivatization with propionic anhydride/benzoic anhydride creates a more hydrophopic, surface active derivative.

Controlling retention of the analytes in the chromatographic system is also an important step when seeking to improve sensitivity (**III**). Signal suppression is often caused by the simultaneous ionization of analytes of interest and interfering substances. Therefore, optimal sensitivity in ESI is obtained if analytes can be eluted completely separately. The signal intensity in ESI is also correlated to the nature of the solvents used. Optimal ionization in ESI is often observed with organic modifier concentrations of around 50% (Cech & Enke, 2001b). Derivatization through propionylation also proved to be excellent for controlling retention and for enabling compounds to be eluted in higher fractions of organic modifier (**III**). The striking effect of derivatization on chromatographic retention is shown in (**III, fig. 4**). Without derivatization, virtually no retention at all was observed, whereas with the derivatization representative bases, ribosides and nucleotides separated very well. A further gain was the demonstrated ability to analyse all major cytokinin metabolites in the same injection. Previous methods have analyzed nucleotides as a separate riboside fraction after de-phosphorylation. Analysis of intact nucleotides simultaneously with other metabolites further increases the speed and potential accuracy of the analysis. The analysis protocol presented in Paper **III** was tested with a real sample and the results for some of the cytokinin metabolites are presented

in (**III, fig. 6**). It is important to be able to perform analysis in as small a sample as possible in order to achieve the highest possible biological resolution. In Paper **III**, we demonstrated the strength of derivatization through either propionylation or benzoylation by analyzing 20 mg samples of *Arabidopsis*. The signal to noise ratios that can be estimated from (**III, fig. 6**) implies that it is probably possible to use this protocol for even smaller samples.

Cytokinin – Auxin Interaction in Arabidopsis mutant, disrupted in the ADK gene (IV)

Paper **IV** details investigations of the cytokinin and auxin contents of a mutant with phenotypic traits of a cytokinin over-producer and an over-proliferating shoot apical meristem (SAM) using methodology developed in **III**. The mutant was found to have a disruption at the *ADENOSINE KINASE1* (*ADK1*) locus. The *Arabidopsis* genome contains two genes encoding the enzyme adenosine kinase, *ADK1* and *ADK2* (Moffat *et al*., 2000). The ADK enzyme is responsible for converting iPA to iPMP as indicated in figure 3. Assays of ADK activity in crude extracts of 3-week-old plants indicated that *adk1* is responsible for 40% of the activity detected in wild type plants (**IV**). The residual ADK activity in the *adk1* mutant is probably due to the presence of ADK2 enzyme that is not affected by the mutation (**IV**). In a root growth experiment where seedlings were grown on hormone-free media for five days before transfer to media containing hormone, *adk1* showed decreased sensitivity towards benzyl amino purine (BAP) (**IV**). The decreased sensitivity was manifested in the form of weaker inhibition of root growth, indicating that the mutant had altered cytokinin levels and/or responses. Cytokinin quantifications revealed a tendency towards increased contents of all measured cytokinins (**IV**). The most pronounced increases were in the ZMP, iPMP, Z7G and Z9G pools and, to a lesser extent, in the ribosides and bases. The ADK enzyme is involved in the conversion of adenosine (Ado) to adenosine monophosphate (AMP), but it has also been proven to convert iPA to iPMP (Moffat *et al*., 2000). To address the question whether the elevated cytokinin levels were due to increased release from conjugated forms or to increased *de novo* cytokinin biosynthesis, we probed the *adk1* and wild type with an *in vivo* deuterium labelling experiment. For iPMP and ZR we detected significantly higher incorporation of ^{2}H and, hence, concluded that the increase in the cytokinin pool was due to an elevation of *de novo* synthesis (**IV**). This is not expected as a direct consequence of the disruption of ADK enzyme activity. There is some enzymatic redundancy in the conversion of bases and ribosides to nucleotides. As well as the ADK converting iPA to iPMP, APT adeninephosphoribosyl transferase has been detected in Arabidopsis (Allen *et al*., 2002). This means that iPMP could be produced via either ADK or APT enzyme activity. If the iPA is first converted to the free base iP it can be subsequently converted directly to iPMP by the action of APT.

It is possible that lower than wild type levels of iPMP occur in the plant due to the disruption of ADK. As a consequence of this iPMP depletion, the *de novo* biosynthesis machinery may be started, causing increased levels of iPMP biosynthesis and pool size. In a further experiment we measured the levels of free IAA and found them to be significantly lower in *adk1* compared to wild type

(**IV**). Could this be related to the findings in paper **II**? We can only speculate. The increased levels of cytokinins were detected at a late developmental stage (we only measured them at one time point). They might arise as a direct consequence of the ADK1 enzyme malfunction or via changes in the growth pattern caused by changes in central metabolites, and then only as a secondary response. Once the cytokinin levels are elevated they might affect IAA biosynthesis in analogy with findings in (**II**). The lowered levels of IAA would then in turn stimulate cytokinin biosynthesis further and so forth in a positive feedback loop. The changed cytokinin/auxin homeostasis could then alter the development of the plant and yield phenotypic traits such as those observed in (**IV**).

Further time-course experiments, or analyses of plants with inducible repression of *ADK1*, could reveal the potential role of the altered cytokinin/auxin homeostasis observed in **IV**. It is difficult to resolve causes and effects of the changed hormone homeostasis and its interrelationships, but using techniques developed in **III** we have observed a changed cytokinin/auxin content ratio and an increase in cytokinin biosynthesis in the described mutant.

Conclusions

From the work performed in this investigation, we would like to propose the following model of cytokinin biosynthesis and cytokinin interaction with auxin:

Cytokinins can be synthesised *de novo* both via iPMP and ZMP. Auxin rapidly regulates cytokinin pools by reducing their biosynthesis. The reduction in biosynthesis is caused by an effect on the ZMP biosynthetic pathway and primarily causes a reduction in the pool of Z-type cytokinins. Normal IAA perception and signal transduction is required for the negative regulatory mechanism to act on cytokinin biosynthesis. Cytokinins regulate IAA biosynthesis and the IAA pool more slowly, probably via developmental changes. Cytokinins can be potentially synthesised throughout the plant with the highest capacity in small leaves and roots. Cytokinins of the iP-type are primarily synthesised in the root whereas Z-types are synthesised in the apical part, as summarized in figure 10.

A mutant affected in the ADK enzyme, displaying phenotypic traits of cytokinin over-production was investigated for auxin and cytokinin content and biosynthesis. Changes in its homeostasis were detected and preliminary results indicate that this might be due to increased *de novo* cytokinin biosynthesis.

Furthermore, a very potent method for cytokinin analysis has been developed. The method can separate and measure, with high sensitivity and accuracy, all major cytokinin metabolites, ranging from free bases to intact nucleotides in minute amounts of plant material (<50 mg). This can be done at high sample throughputs (≈70 samples/day).

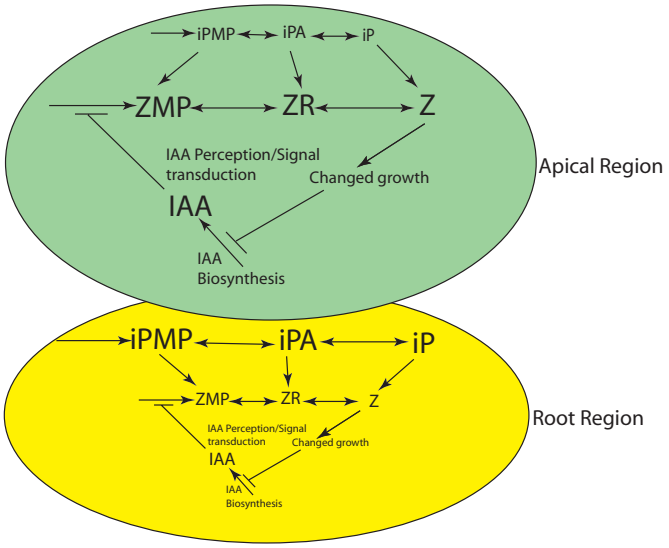

Figure 10. Model: both ZMP and iPMP are potentially synthesized *de novo*. IAA negatively regulates cytokinin biosynthesis through IAA signal transduction in the *de novo* ZMP biosynthesis pathway. ZMP is predominantly synthesized in the apical part, whereas iPMP is predominantly synthesized in the root region. The capacity to synthesise cytokinins de novo is present in both the apical and the root region.

Further Perspectives

Many details of the molecular basis of cytokinin biosynthesis and perception have been discovered during the last few years. Endogenous *Arabidopsis* cytokinin biosynthesis genes have been cloned and cytokinin receptors have been identified. On the metabolite side, however, many questions remain to be answered. Answering some of these questions will require:

- Identification of the HMBPP metabolite *in planta*
- Unambiguous identification of the first cytokinin biosynthetic products: ZTP, ZDP, iPTP and iPDP *in planta*
- Comparison of the relative importance of the biosynthetic genes AtIPT1 through 9 for the synthesis of Z and iP type cytokinins, respectively, through *in vivo* deuterium feeding experiments.

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