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Mechanism of cytokinin transport across plasma membrane and their metabolism in tobacco BY-2 cultured cells

Ph.D. Thesis

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This is to certify that this thesis is not a subject of any other defending procedure. It contains a set of original results that have been published or are going to be published in the international peer-reviewed scientific journals.

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Contents

Abbreviations

Ade	adenine
Ado	adenosine
Arabidopsis	*Arabidopsis thaliana* (L.) Heynh.
BA	N^6-benzyladenine
BA7G	N^6-benzyladenine 7-glucoside
BA9G	N^6-benzyladenine 9-glucoside
BAR	N^6-benzyladenine 9-riboside
BARMP	N^6-benzyladenine 9-riboside-5'-monophosphate
BY-2	*Nicotiana tabacum* L. cv. Bright Yellow 2 cell culture
CCCP	carbonylcyanide *m*-chlorophenyl hydrazone
CK	cytokinin
CKX	cytokinin oxidase/dehydrogenase
CNT	concentrative nucleoside transporter
cZ	*cis*-zeatin
DHZ	dihydrozeatin
DHZR	dihydrozeatinriboside
DMSO	dimethyl sulfoxide
ENT	equilibrative nucleoside transporter
FW	fresh weight
GUS	β-glucuronidase
iP	N^6-(Δ^2-isopentenyl)adenine
iP7G	N^6-(Δ^2-isopentenyl)adenine 7-glucoside
iPR	N^6-(Δ^2-isopentenyl)adenine 9-riboside
iPRMP	N^6-(Δ^2-isopentenyl)adenine 9-riboside-5'-monophosphate
IPT	isopentenyltransferase
K_d	displacement constant
K_i	inhibition constant
K_M	Michaelis constant
Kin	kinetin
PUP	purine permease
tZ	*trans*-zeatin
tZR	*trans*-zeatin 9-riboside
tZRMP	*trans*-zeatin 9-riboside-5'-monophosphate

1. Introduction

1.1 The pioneers

Cytokinins (CKs) are plant hormones that play a decisive role in many aspects of plant development including germination, root development, shoot growth, seed size and leaf senescence (Riefler et al. 2006), nodulation (Tirichine et al., 2007), circadian rhythms (Hanano et al., 2006) and stress responses (reviewed by Hare et al. 1997 and Argueso et al. 2009). An intriguing fact is that CKs, from the dawn of plant growth regulators research, are tightly linked to other plant hormones, especially auxin. Thanks to advances in genomics and proteomics, completion of sequencing of genomes of several plant species, availability of loss-of-function mutants and accessibility of massive screening methodology, it became recently possible to uncover molecular basis not only of CK mode of action but also of their interplay with other stimuli.

CKs have been discovered during the quest for cytokinesis promoting factors in plant cell culture research during the 1950's. The pioneering work was done by Professor Folke Skoog and his co-workers at the University of Wisconsin, USA. In the course of 1940's, *in vitro* plant aseptic tissue culture was gradually established. The basic compounds of the growing media (sugar, minerals, vitamins, auxin) were already known but with one particular exception: an undefined chemical substance which needed to be supplied through the means of autoclaved coconut milk for example (Van Overbeek et al. 1941).

Miller et al. (1955), finally succeded to isolate the active purine-like compound from autoclaved herring sperm DNA. In cooperation with Prof. Strong's laboratory the substance was identified, by procedures including synthesis, as 6-furfurylaminopurine (Miller et al. 1956). Subsequent tests have shown the ability of the compound to promote cell division even in a very low concentration. Therefore it was given the trivial name of kinetin (a subtle suggestion how to pronounce the word was provided as well: "kine'-uh-tin", sic) and the term "kinins" was proposed to be the class name to cover substances with similar effect, from which diphenylurea has been known at that time (Shantz and Steward, 1955). "Thus, kinetin is one specific kinin, as indoleacetic acid is one specific auxin" wrote Miller to introduce a new class of phytohormones that promote cytokinesis (Miller et al. 1955; Miller et al. 1956). The name kinin was later

changed to cytokinin to prevent confusion with another class of compounds stimulating muscle contraction in animal systems (Skoog and Armstrong, 1970).

Still, kinetin was thought to be an artifact arised spontaneously from DNA preparation, so the race for discovery of natural compound was started soon after its finding. Miller himself attempted to isolate a natural cytokinin from immature maize seeds. Eventhough he purified the extract to a high degree, he was not successful with crystallization, thus failing to provide the actual chemical formula of the factor (Miller et al., 1956). Therefore, it was Letham et al. (1963) who claimed the right to chemically characterize and name the first naturally occurring cytokinin prepared from the same starting material: zeatin from immature grains of *Zea mays*. In his paper, he states that Miller's compound is probably identical and that zeatin might be a growth regulator of wide occurrence. Moreover, Professor Folke Skoog, in his later memories (Skoog, 1994) and contrary to usual textbook statements, claims that Miller should probably be given priority for isolation and composition of zeatin.

1.2 Inspirations for my thesis and personal overview

During my master thesis I worked on cytokinin and *Kn1*-family homeogenes interplay in *Arabidopsis* in the Laboratory of Molecular Plant Physiology at the Faculty of Science of the Masaryk University in Brno, under supervision of Assoc. Prof. Dr. Břetislav Brzobohatý. For my doctoral studies I joined the research group of Assoc. Prof. Dr. Eva Zažímalová in the Laboratory of Hormonal Regulations in Plants at the Institute of Experimental Botany, and stayed with cytokinins.

In 2003, auxin transport assays performed on tobacco BY-2 cell culture were already a well established laboratory's own technique and we agreed that BY-2 suspension cells would be a suitable tool to assess CK transport on the cellular level of plants. Moreover, our laboratory had a wide and solid CK-related scientific background ranging from CK metabolism, through hormonal interplay to stress biology. Not to mention the instrumentation and methods available and… Dr. Mirek Kamínek's work experience from Folke Skoog's lab. I also had a great review in hands that I find stimulating up to now: Miroslav Kamínek, Václav Motyka & Radomíra Vaňková: Regulation of cytokinin content in plant cells, 1997.

CK transport was studied from late 1960's by Lagerstedt and Langston (1967) on bean leaves vasculature or leaf discs. Also, first essays performed with suspension cells of *Acer pseudoplatanus* recorded the uptake and metabolism of CKs (Dorée and Guern, 1973). Further CK transport experiments were succesfully made with tobacco cell culture by Prof. Michel Laloue

and his group and date from late 1970's. Their research represents one of the first attempts to connect CK uptake and metabolism on the cellular level and was one of the main inspirational sources for me.

When I started my work on CK accumulation in BY-2, the group of Dr. Wolf Frommer had published two papers describing the energy dependent candidates for CK transporters, members of the purine permease family. During my work in 2005, other candidate proteins for CK transporters, members of the family of plant equilibrative nucleoside transporters from *Arabidopsis* and rice, were announced by the group of Prof. Sakakibara. At that time also, papers by Dr. Petr Mlejnek brought a surprising evidence for CKs acting like apoptosis inducers when applied in high concentration to BY-2 cells. Needless is to say that all these facts represented a great source of challenge and inspiration at the same time, and were a basic motivation for competition studies on BY-2 cell suspension.

In 2005 first results entitled "*The mechanism of accumulation of cytokinins in suspension-cultured tobacco cells*" were presented on a poster at ACPD international conference in Prague. A diploma student and a CK-transport colleague of mine, Libuše Marková (Bartošová), successfully defended her diploma work on CK transport the same year.

As a member of "The auxin group" of our laboratory, I did not escape auxin. A side project arose from the collaboration with my former colleagues from Brno, who explored the molecular basis of auxin-cytokinin interaction. I was involved in testing the influence of CKs on the efflux of NAA (1-naphthaleneacetic acid). The paper is included in my publication list but is not subject of this thesis which concentrates solely on CK transport.

Later I proceeded with metabolic profiling with the help of Dr. Klára Hoyerová and Dr. Petre I. Dobrev who both stay behind the development and optimalization of the methods for isolation and detection of CKs (Dobrev and Kamínek, 2002; Hoyerová et al., 2006). Motivated by the results of these experiments, CKX assay was performed with the help of Dr. Václav Motyka who modified and optimized the method together with Dr. Mirek Kamínek (Motyka and Kamínek, 1994).

Some of the results were presented as posters in Prague at ACPD conference in 2009 ("*Transport of cytokinins in BY-2 suspension-grown tobacco cells – towards a mathematical model*") and in Tarragona, Spain at the IPGSA 2010 international conference ("*After uptake – A fast metabolism of cytokinins in BY-2 tobacco cells*"). I also contributed to the paper by Gajdošová et al. (2011) with testing the transport characteristics of cZ and tZ in BY-2 cell culture

and participating on short-term metabolism comparison of cZ and tZ in BY-2. The paper is included in this thesis.

To my knowledge, no similarly extensive set of data describing the competition of CK-bases and CK-ribosides in plant cells has been presented so far. Use of radiolabelled cytokinin substrates allowed me to connect the uptake and subsequent metabolism of these signalling molecules. In plants, the two processes seem to be inseparably linked and thus potentially constitute a basic principle of CK action. I am trying to discuss this issue.

2. Abstract

Cytokinins (CKs) are plant hormones that play a major role in a number of developmental processes in plants. Those include promotion of cell division, active growth and differentiation, and maintenance of sink-source relationships, as well as control of environmental stress responses. Native CKs are low-molecular derivatives of adenine which seem to act either as paracrine or as long-distance signals. Due to their numerous physiological effects, plants have to precisely control the occurrence of bioactive CK molecules on the levels of the whole plant, its organs, tissues as well as single cells. To achieve this, a concerted action of metabolism and transport processes is required.

Studies of the kinetics of CK translocation across plasma membrane in BY-2 suspension-grown tobacco cells suggested the existence of energy-dependent, partially selective transport routes for CK bases and CK ribosides. HPLC analysis of the metabolites of accumulated CKs pointed at their fast degradation or metabolic conversion into physiologically inactive forms. The prevalent ways of inactivation were the degradation to adenine and phosphorylation or phosphoribosyl transfer to form the nucleotide relevant to the particular, [^{3}H]-labelled CK. Analysis of crude extracts of CK oxidase/dehydrogenase enzymes from BY-2 revealed their highest affinity towards isopentenyladenine and found their activity dependent on concentration of externally supplied CK.

Together these results suggest a close link between the ability of plant cell to transport CKs across plasma membrane and its capability of a prompt metabolic inactivation of these signalling molecules. They also highlighted the rapidity and robustness of metabolic changes in cells related to CKs.

3. Research objectives

The objective of this thesis is to contribute to elucidation of CK transport at the plant cellular level. Such data are scarce up to now. Eventhough the candidates for CK transmembrane carriers were identified, their biochemical characterization has been made predominantly on heterologous systems such as yeast. In this study I try to describe the characteristics of accumulation of selected [^{3}H]-labelled bioactive cytokinins and address the question of existence of two distinct transport systems for CK-bases and CK-ribosides using BY-2 suspension-grown tobacco cells, i.e. a purely *in planta* system. In our laboratory, tobacco BY-2 cell line (Nagata et al. 1992) has been a well-established model for measuring of auxin transport (Petrášek et al., 2006; Petrášek & Zažímalová, 2006).

In summary this thesis is focused on the following key points:

- checking the usability of BY-2 tobacco suspension culture for CK transport studies
- describing the kinetics of short-term CK uptake
- probing the existence of selective transmembrane transport routes for CK-bases and CK-ribosides
- testing the energy dependence of CK transport
- describing the intracellular metabolic fate of uptaken CKs

4. Literature overview

4.1 Chemical structure of cytokinins

Chemically, cytokinins are derived from adenine with a substituted N^6 side chain (**Fig 1**). The structural variance of this side moiety as well as metabolic changes on the adenine ring result in a wide range of chemical substances within the cytokinin metabolic network that possess different CK activities. According to the character of the side chain, naturally occurring CKs can be divided in two main subgroups - isoprenoid and aromatic CKs. In general, a specific plant tissue contains several types of CKs and their modified forms. The distribution of various CKs is depends on plant species and developmental stage, however CKs with unsaturated isoprenoid side chain, *trans*-zeatin (tZ), isopentenyl adenine (iP) and *cis*-zeatin (cZ), together with saturated dihydrozeatin (DHZ) seem to be the most prevalent in the plant kingdom (Sakakibara 2006). Aromatic CKs (Strnad, 1997), namely *ortho*- and *meta*-topolin, their methoxy-derivatives and benzyladenine, have been found so far only in some plant species like tomato (Nandi et al. 1989), poplar (Horgan et al. 1975; Strnad et al. 1997) and *Arabidopsis* (Tarkowská et al. 2003) for example. Recently, the role of *cis*-zeatin, reportedly a physiologically inactive zeatin isomer, is being reassessed as relatively high portions of cZ are found in some plant species (Gajdošová et al. 2011). Interestingly, the long lasting conviction about the unnatural origin of kinetin was doubted recently (Barciszewski et al. 2007).

The majority of CKs in plants exist in a free, soluble form. Besides, some cytokinin nucleotides were found as structural components of certain tRNAs of domains of Eucarya and Bacteria (but not Archea), including plants and animals, where they are found adjacent to the 3'-end of the anticodon, possibly stabilizing the codon-anticodon binding (Pačes & Kamínek 1976; Bouadloun et al. 1986; Persson et al. 1994). In addition, a group of synthetic diphenylurea derivatives, structurally unrelated to adenine-type CKs, also possess CK activity (Shantz and Steward, 1955).

4.2 Metabolism of cytokinins

4.2.1 Biosynthesis of cytokinins

Due to their presence in tRNA, CKs were thought to originate from its breakdown. However, it has been suggested that tRNA degradation may account for maximally 40 % of total free CKs detected in plants (Barnes, 1980). Moreover, tRNAs of higher plants contain cZ exhibiting much lower CK activity than the corresponding *trans*-isomer (Kamínek et al. 1979) and may restrict

interference of tRNA CKs in hormonal regulations (Kamínek 1974). Since isopentenyl-derived CKs are prevalent in plants, the main focus was brought on the iP-type rather than aromatic CK biosynthesis. Actually, the biosynthetic pathway of aromatic cytokinins has not been identified so far.

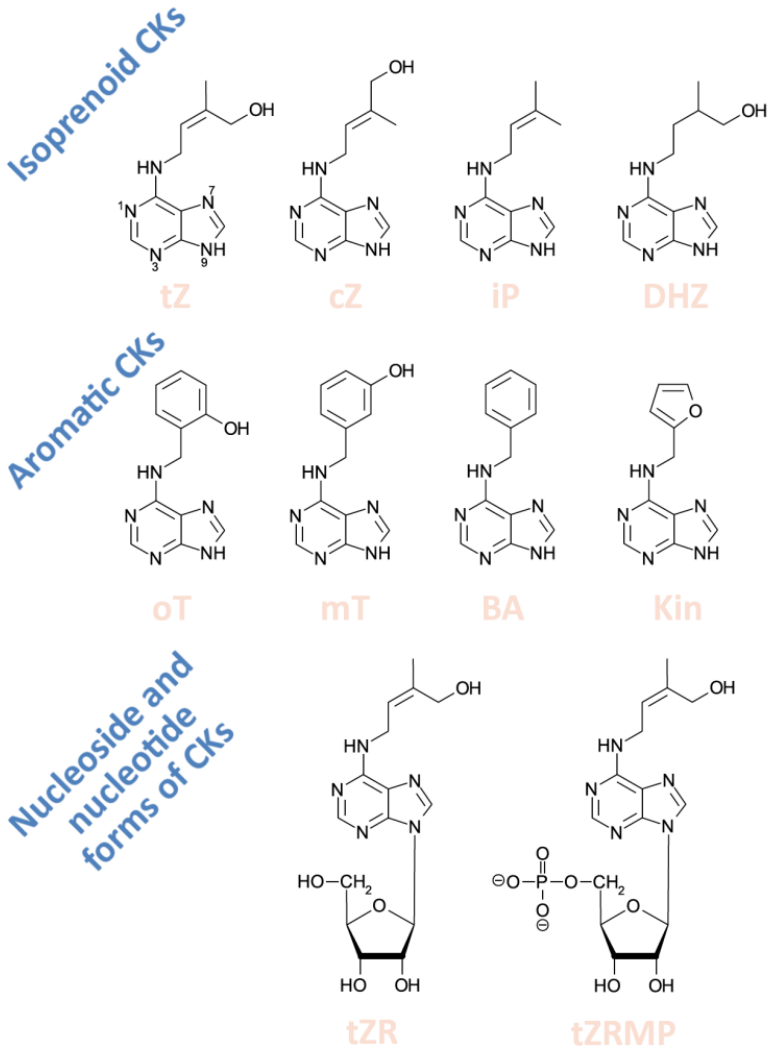

Figure 1 Structures of representative cytokinin species occuring naturally: *trans*-zeatin (tZ), *cis*-zeatin (cZ), isopentenyladenine (iP), dihydrozeatin (DHZ), *ortho*-topolin (oT), *meta*-topolin (mT), benzyladenine (BA).

Kinetin (Kin) was the first cytokinin discovered and for a long time was considered to be an artifact of unnatural origin.

Nucleoside and nucleotide forms of CKs are represented by *trans*-zeatin riboside (tZR) and *trans*-zeatin riboside monophosphate (tZRMP), respectively.

Isopentenyltransferase (IPT) catalyzing the key step in biosynthesis of CKs has been known from *Dictyostelium discoideum* (Taya et al. 1978). Nevertheless the first isolated *IPT* genes were encoded by *Tmr* (tumor morphology „rooty“) locus of T-DNA of *Agrobacterium tumefaciens*

(Akiyoshi et al. 1984; Barry et al. 1984) and by its *Tzs* (*trans*-zeatin secretion) locus of the Ti plasmid outside of T-DNA (Beaty et al. 1986). With the advancement of genome sequencing projects on *Arabidopsis* and rice, complete gene families for plant isopentenyltransferases have been isolated (Kakimoto 2001; Takei et al. 2001; Sakamoto et al. 2006). There are seven *IPTs* in *Arabidopsis* (*AtIPT1*, *3-8*) and 8 in rice (*OsIPT1-8*) that catalyze the *N*-prenylation step in CKs biosynthesis. AtIPT2 and AtIPT9 of *Arabidopsis* act as tRNA-IPTs (Miyawaki et al., 2006). Unlike bacterial Tmr, AtIPTs and OsIPTs prefer ADP and ATP over AMP as prenyl acceptor, and dimethylallyl diphosphate (DMAPP) over 4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBDP) as the prenyl donor (Sakakibara et al. 2005). DMAPP originates either from the methylerythritol (MEP) pathway in plastids or from the mevalonate (MVA) pathway in the cytosol (Kasahara et al. 2004). The former pathway provides DMAPP mainly for biosynthesis of free bioactive cytokinins while the latter for prenylation of tRNAs (Kasahara et al. 2004; Sakakibara 2006).

The reaction between DMAPP and ADP or ATP produces iP-nucleotides, specifically iP riboside 5'-diphosphate (iPRDP) and iP riboside 5'-triphosphate (iPRTP) (Kakimoto 2001; Sakamoto et al. 2006) which undergo further metabolic conversions. Interestingly, Astot et al. (2000) proposed an alternative, iPMP-independent pathway where 4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBDP) serves as a hydroxylated side chain donor, leading directly to *trans*-zeatin.

4.2.2 Interconversions

Being derived from purine, some of the reactions associated with CKs are common for the general purine metabolism while some are supposed to be specific for CKs. After the initial isopentenylation, iPRTP or iPRDP undergo a series of dephosphorylations that lead to isopentenyladenosine monophosphate (iPRMP). During all dephosphorylation steps, a side chain hydroxylation by a CK *trans*-hydroxylase may occur to give an appropriate *trans*-zeatin phosphoderivate. Two CK *trans*-hydroxylases (CYP735A1 and CYP735A2) that belong to a family of cytochrome P450 monooxygenases, have been identified in *Arabidopsis* (Takei et al. 2004).

Currently, two ways for generation of a free cytokinin base from a 5'-monophosphate are known. First is the two-step enzymatic reaction with dephosphorylation to a riboside followed by hydrolysis of the riboside intermediate, second is the one-step catalysis by LOG phosphoribosylhydrolase (*lonely guy* mutation gives only one pistil and no stamen in rice flowers) discovered in *Oryza sativa* (Kurakawa et al., 2007). A family of nine LOG homologs has been detected in *Arabidopsis* (Kuroha et al., 2009). However, reported opposite conversion of CK

bases to the corresponding ribotides by adenine phosphoribosyltrasferase (Chen and Petschow, 1978) indicates that the product can serve as a CK storage compound.

Saturation of the tZRMP, tZR and tZ side chains by a reductase may lead to the formation of corresponding dihydrozeatin derivates, but the conversion has been shown only for tZ to DHZ so far (Martin et al. 1989).

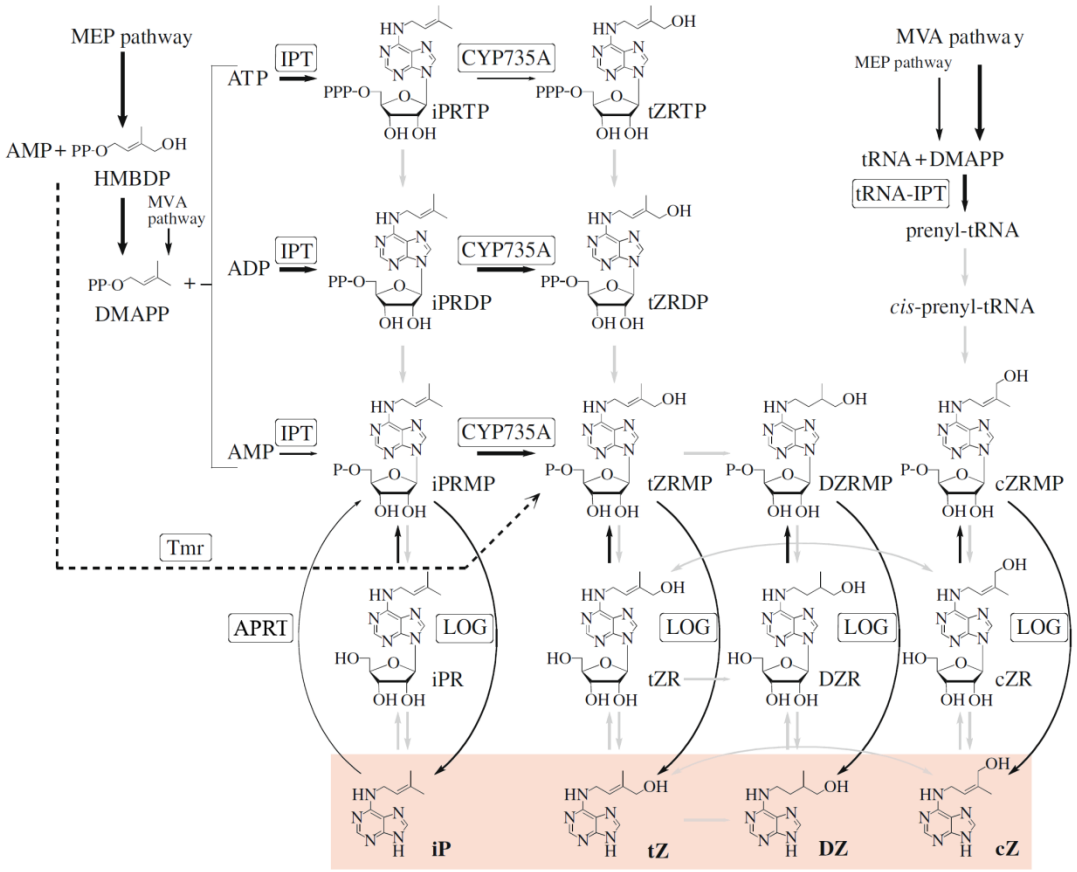

Figure 2 Current model of isoprenoid CK biosynthetic pathways and conversions in higher plants and plant cells infected by *Agrobacterium tumefaciens*. The model includes the two possible source pathways generating DMAPP - the prenyl donor for IPT-mediated isopentenylation of ATP, ADP or AMP; as well as dephosphorylation cascade and hydroxylation of its iP-intermediates by CYP735A, leading to iPR- and tZR-monophosphates. A one-step (catalyzed by LOG) or two-step (dephosphorylation followed by deribosylation) paths lead to bioactive CK bases. The dashed arrow line depicts the prenylation of AMP by HMBDP producing tZRMP in cells infected by *A. tumefaciens*. A *cis-trans* isomerase may balance the cZ/tZ ratio. Adenine phosphoribosyltransferase (APRT) utilizes not only iP but also other nucleobases as substrate. The width of the lines indicates the strength of the metabolic flow and gray arrows show enzymes not yet identified. (Modified from Kamada-Nobusada and Sakakibara 2009).

Metabolic origins of *cis*-zeatin seem to be specific. An isomerisation from tZ or tZR is likely to occur although a spontaneous isomeration seems unlikely for thermodynamic reasons and the enzyme has not yet been fully characterized in plants (Gajdošová et al. 2011). On the other hand,

the *cis-trans* isomerase activity has been detected in immature seeds of Phaseolus (Bassil et al., 1993). In *Arabidopsis*, biosynthesis of cZ requires the activity of tRNA-IPTs (AtIPT2 and 9) (Miyawaki et al., 2006) and the cytosolic, MVA pathway-derived source of DMAPP (Kasahara et al., 2004) .

4.2.3 Conjugation and degradation

Degradation, inactivating conjugation and release of bioactive CK nucleobase from conjugates are means of regulation of the levels of bioactive CKs in plants. *N*-glucosylation affects the adenine ring at the 3-, 7- or 9-position. So far, 3-*N*-glucosides of cytokinins have been detected only in some plants such as radish (Letham et al. 1975). Five glycosyltransferases have been identified in *Arabidopsis* to recognize CKs, from which two (UGT76C1 and UGT76C2) are *N*-glucosyltransferases preferring to glucosylate the N7 position to N9. None of them produced 3-*N*-glucosides though (Hou et al. 2004). Similarly, *O*-glucosylation or *O*-xylosylation have been observed at the hydroxyl group of the side chain of tZ, cZ and topolin (preferentially *o*- and *m*-topolin) and the enzymes have been characterized (Martin et al. 1999a; 1999b; 2001; Veach et al. 2003; Mok et al. 2005). CK bases can be released from *O*-glucosides by β-glucosidase whereas *N*-glucosylation is practically irreversible (Brzobohatý et al. 1993). Therefore, it has been suggested that *O*-glucosides may represent a readily available but biologically inactive storage form of CKs.

A cleavage of the unsaturated isoprenoid side chain is catalyzed by CK oxidase/dehydrogenases (CKX). The enzyme specificity is aimed towards iP, tZ and their ribosides and cZ while cZR, DHZ, Z-9-glucoside and BA are not substrates (Bilyeu et al., 2001; Frébort et al., 2002). The first CKX activity in plants has been reported in crude tobacco tissue extract (Pačes et al., 1971). Since then, CKX enzymes have been characterized in a number of plant species and, similarly to IPT, the CKX activity has been identified in *Dictyostellium discoideum* and in several species outside of green plants (for review see Galuszka et al. 2000). *Arabidopsis* and rice genomes harbour seven (*AtCKX1-7*) and up to eleven (*OsCKX1-11*) CKX genes, respectively (Schmülling et al. 2003; Werner et al. 2006). AtCKXs are specific in their biochemical properties and subcellular localization: AtCKX1 and 3 are targeted to vacuole (Werner et al. 2003), AtCKX7 is restricted to the cytosol, whereas AtCKX2 and AtCKX4-6 are probably secreted (Schmülling et al. 2003). Interestingly, CKX activity is enhanced by application of exogenous isoprenoid CKs as well as by those which do not serve as substrates, like aromatic cytokinins and thidiazuron (Chatfield and Armstrong, 1986) and by endogenous iP, following enhanced *IPT* expression (Werner et al. 2001)

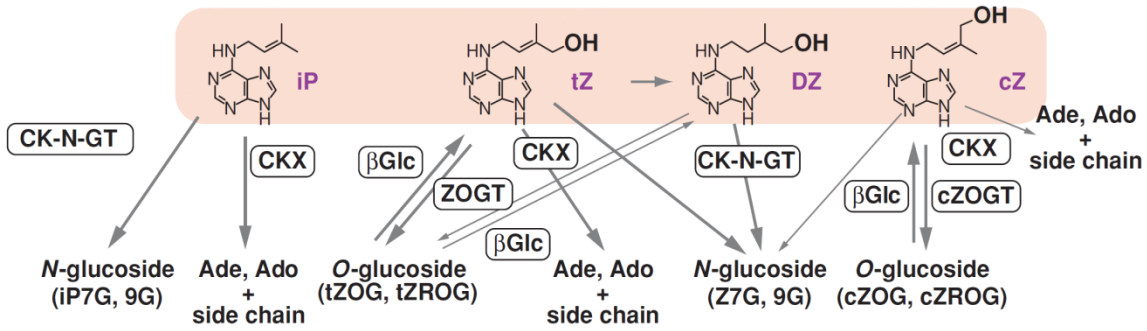

Figure 3 Schematic view of enzymatic degradation and conjugation of bioactive CKs. iP, tZ and cZ and their corresponding nucleosides (not shown) can be catabolized by CKX to adenine (Ade) or adenosine (Ado). tZ can be reversibly converted to *O*-glucoside by zeatin *O*-glucosyltransferase (ZOGT) and by β-glucosidase (βGlc). CK bases can also be converted to *N*-glucosides by CK *N*-glucosyltransferase (CK-N-GT). The width of arrows indicates the strenght of metabolic flow. (Adapted from Sakakibara 2006).

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4.3 Cytokinins as a local or a long range signal

4.3.1 Evidence for cytokinins as a local signal

CKs have been usually thought to be synthesized in roots and transported to the shoot with transpiration flow through xylem. However, the spatial expression patterns of *IPTs* in *Arabidopsis* (Miyawaki et al. 2004; Takei et al. 2004) show that CKs biosynthesis sites are more diverse than that. *AtIPT1* is expressed in root tips, leaf axils, ovules and immature seeds; *AtIPT3* in phloem companion cells; *AtIPT4* in immature seeds; *AtIPT5* is expressed in lateral root primordia, columella root cap, young inflorescence and fruit abscission zone; *AtIPT6* in siliques; *AtIPT7* in phloem companion cells, endodermis of the root elongation zone, trichomes of young leaves and pollen tubes and *AtIPT8* shares the pattern with *AtIPT4*.

Such a spatial expression diversity of CK biosynthetic genes indicates that CKs can be locally synthesized and act as autocrine or paracrine signal. Indeed, earlier reports pointed to the local CK action demonstrating that attraction of metabolites in leaves and shoot budding in calli are exhibited directly under the localized source of externally supplied hormone (Grayburn et al. 1982). Similarly, a conditional induction of *Agrobacterium IPT* leads only to a local lateral bud outgrowth in tobacco as shown by Faiss et al. (1997). Moreover, after decapitation, local CK biosynthesis is induced in nodes of *Pisum sativum* rather than in roots (Tanaka et al. 2006). The importance of local active CK supply has been confirmed as well in *log* mutant plants where the release of free cytokinin base from its nucleotide form is disrupted (Kurakawa et al., 2007).

4.3.2 Long range aspect of cytokinin signalling

On the contrary, if we borrow another piece of terminology from animal hormonal research, CKs possess also an "endocrine" aspect in their function. Endocrine animal hormones are secreted to the blood stream and transported to a distant organ to exert their function. By analogy, various CKs are known to represent a long range mobile signal which is transported via vasculature by xylem and phloem streams. CKs presence in xylem and or phloem saps has been detected in multiple plant species including sunflower (Bano et al., 1994), pea (Beveridge et al., 1997), cotton (Yong et al., 2000), maize (Takei 2001), cucumber (Kuroha et al., 2002), tomato (Kudoyarova et al., 2007) and *Arabidopsis* (Corbesier et al., 2003; Foo et al., 2007; Hirose et al., 2008).

Up to date, major CKs in the xylem sap are the tZ and its bioactive metabolites with tZR being the most abundant. Together with the predominant expression pattern of the CYP735A2 in roots (Takei et al., 2004), these results point to roots as the main site for tZ biosynthesis in plants and to the role of tZR in root-to-shoot signalling. The CK dependent long range signalling has been linked to soil nutrient availability, namely nitrogen in barley (Samuelson et al. 1992) and in maize (Takei et al. 2001). After nitrate application, *AtIPT3* expression is induced in *Arabidopsis* roots, followed by an increase of tZ-ribotides and tZR (Takei et al. 2004). Transpiration-dependent xylem flow of CKs has been demonstrated in *ARR5::GUS* expressing plants by Aloni et al. (2005).

4.4 Cytokinin signal transduction

4.4.1 Cytokinin receptors

Today, CK signalling pathway in plants is relatively well uncovered. The current model is considered to be a multistep phosphorelay pathway similar to bacterial two-component signalling system (To & Kieber, 2008; Perilli et al., 2010). In *Arabidopsis*, three transmembrane hybrid histidine kinases, AHK4 (ARABIDOPSIS HIS KINASE 4, independently isolated as WOL1 [WOODEN LEG 1; Mähönen et al., 2000] and CRE1 [CK RESPONSE 1; Inoue et al., 2001]) and its homologs AHK2 and AHK3 receive the CK signal at the N-terminal CHASE (cyclase/histidine-kinase-associated extracellular) domain. After autophosphorylation at the cytoplasmic His kinase domain of the receptor, the phosphoryl group is transferred to the conserved Asp residue on the receptor's receiver domain. The resulting CK-dependent kinase activity targeted on HPts (histidine phosphotransfer proteins) was recorded in heterologous bacterial and yeast systems (Suzuki, 2001; Ueguchi, 2001; Yamada, 2001).

4.4.2 Downstream signal transduction

In *Arabidopsis*, posphorylation downstream of AHK receptors is mediated by 5 members of ARABIDOPSIS HIS PHOSPHOTRANSFER PROTEIN (AHP) family (Hutchison et al. 2006). AHPs bring the signal to nucleus and phosphorylate type-A ARRs (ARABIDOPSIS RESPONSE REGULATORs), which act as negative regulators of CK responses, and type-B ARRs proteins, which are transcription factors with positive role in CK-regulated gene expression. At the same time and independently of ARRs, CRFs (CYTOKININ RESPONSE FACTORS) accumulate in the nucleus as a result of CK-linked signalling, and act in concert with ARRs to mediate the CK response (Rashotte et al. 2006).

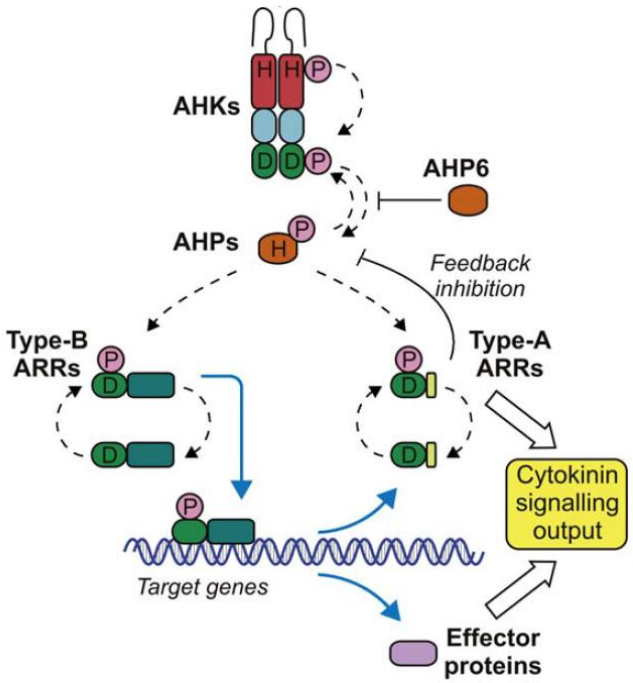

Figure 4 Schematic model of the core steps of the cytokinin signalling pathway. A His(H)/Asp(D)-phosphorelay (indicated by dashed arrow lines) is activated by CK binding to the CHASE domain of the histidine kinase receptors (AHKs). After autophosphorylation at the kinase domain (red) of the receptor, the phosphoryl group (rose) is transferred to the receptor receiver domain (green). The signal is further propagated by a family of conserved histidine phosphotransfer proteins (AHPs, orange). AHPs transfer the signal to the nucleus and activate the CK response via type-B response regulator (ARR) transcription factors. A negative regulation of the cascade is provided with type-A ARRs. AHP6 inhibits the phosphoryl transfer and a non-acivated CRE1/AHK4 receptor possesses phosphatase activity that dephosphorylates AHPs. (Modified from Werner and Schmülling 2009).

A negative regulation is also inherent to CK phosphorelay signalling pathway. First, at the level of receptors, at least AHK4 is known to behave bidirectionally in vitro, in yeast and in planta. Not only AHK4 positively mediates the CK signal in case of CK presence, but acting as a phosphatase it is able to dephosphorylate HPts in the absence of CK (Mähönen et al. 2006). Secondly, a sixth member of the *Arabidopsis* HPt family, AHP6, has been shown unable to participate in phosphotransfer due to lack of conserved His residue, but rather to counteract CK signalling. The ahp6 mutation has potential to partially revert the wol mutation of AHK4 receptor and confers increased CK sensitivity to CK (Mähönen et al. 2006). Lastly, a negative feedback is assured by the action of type-A ARRs which are rapidly induced in response to CK by action of type-B ARRs (To et al. 2007). While the molecular mechanism of type-A ARRs action is still unknown, phosphorylation is required for their action and a subset of these stabilize in response to CK (To et al. 2007).

4.4.3 Genome-wide cytokinin response

At the genome-wide level, expression screen performed on 8300-element Affymetrix *Arabidopsis* GeneChips revealed at least 30 genes whose expression raised and at least 40 that were down-regulated after application of exogenous tZ and BAP. Among the up-regulated genes were type-A ARRs, cytokinin oxidase/dehydrogenase and several other transcription factors. Down-regulated genes included several peroxidases and E3 ubiquitin ligase (Rashotte et al. 2003).

A screen by Hoth et al. (2003), performed with MPSS (massively parallel signature sequencing) identified 823 genes up-regulated and 917 genes down-regulated 24 hours after induction of bacterial *IPT* in *Arabidopsis thaliana*. When comparing CK response 6 and 24 hours after *IPT* induction, the study found different clusters of genes showing similar course of regulation.

The most detailed experiment with Affymetrix ATH1 gene-chip covering about 23750 loci identified 71 up- and 11 down-regulated genes 15 minutes after CK treatment of *Arabidopsis* seedlings. Amongst were a large portion of transcriptional factors, some of them previously not connected to CKs, and a set of plastid genes as well. 2 hours long treatment pointed to a delayed transcriptional increase of several groups of genes including transcription factors and genes related to signalling, metabolism, hormonal regulation, energy generation and stress reactions (Brenner et al., 2005).

4.5 Transmembrane transport of cytokinins

Due to distribution of CKs between xylem and phloem, it is conceivable that a selective transport systems exist in plants that distribute CKs appropriately. Indeed, members of *Arabidopsis* PURINE PERMEASE and of *Arabidopsis* and rice EQUILIBRATIVE NUCLEOSIDE TRANSPORTER families have been shown to be able to transport free CK-bases and CK-ribosides, respectively.

4.5.1 Purine permeases (PUPs)

15 *PUPs* have been found in the *Arabidopsis* genome (Gillissen et al., 2000), by a later analysis the number was corrected to 21 (Cedzich et al., 2008). They encode for small, hydrophobic integral membrane proteins that mediate the transport of purine derivatives. In yeast, AtPUP1 has been shown to transport adenine, cytosine and some other purine-derived compounds like tZ, kinetin and caffeine (Gillissen et al. 2000). Similarly, AtPUP1 and AtPUP2, but not AtPUP3, mediated uptake of adenine in yeast and a competitive inhibition by BA, *trans*- and *cis*-zeatin was observed. Direct measurement revealed that radiolabelled ^{3}H-*t*Z can be taken up by yeast and *Arabidopsis* suspension cells. The promoter-reporter fusion with *GUS* gene indicated that

AtPUP1 is expressed in the epithem of hydathodes and at the stigma surface of siliques, *AtPUP2* in phloem cells of leaves and *AtPUP3* in pollen of *Arabidopsis* (Bürkle et al. 2003). Cedzich et al. (2008) measured radiolabelled tZ uptake in *Arabidopsis* cell culture. They found that the kinetics of uptake is multiphasic, and involves both low- and high-affinity CK transport systems. In addition, the protonophore carbonyl cyanide m-chlorophenylhydrazone effectively inhibited cytokinin uptake, which was consistent with previous hypothesis of proton-driven, PUP-mediated uptake on yeast (Bürkle et al. 2003).

4.5.2 Equilibrative nucleoside transporters (ENTs)

By prediction from genome sequence, the equilibrative nucleoside transporter family in *Arabidopsis* comprises 8 potential genes that encode for polypeptides strongly similar to ENTs from other organisms (Hyde et al. 2001). Molecular and biochemical analysis of AtENTs revealed that AtENT1, 3, 4, 6 and 7 promote adenosine and some other nucleosides import in yeast cells and that the uptake mechanism differs in proton dependency (Möhlmann et al. 2001; Li et al. 2003; Wormit et al. 2004).

There is a certain confusion about ENTs nomenclature. The protein family name reflects the properties of prototypical human hENT1 which indeed was an equilibrative nucleoside transporter. Many members of the ENT family behave more like concentrative nucleoside transporters (CNT) that are dependent on symport of Na^+ or H^+. This appears also to be the case of ENTs of higher plants. (Hyde et al., 2001).

In *Arabidopsis* suspension cells, the transcription of *AtENT1*, *3*, *4*, *6* and *8* increased after inhibition of *de novo* nucleotide synthesis, indicating that some of the members of AtENT family may function in the salvage pathway of nucleotide synthesis (Li et al. 2003). Further, the expression patterns of different *AtENTs* showed varying degree of tissue specificity. However all *AtENTs* were expressed in leaf and flower (Li et al. 2003). Finally, AtENT1, 3 and 6 were targeted to plasma membrane in homologous and/or heterologous arrangement (Li & Wang 2000; Li et al. 2003; Wormit et al. 2004).

So far, CK transport activity was proposed for AtENT3 and 8 (Sun et al. 2005), AtENT3, 6 and 7 (Hirose et al. 2008) in *Arabidopsis* and OsENT2 in rice (Hirose et al. 2005). In *Arabidopsis*, a loss-of-function mutation in *AtENT8* or *AtENT3* causes a decrease in sensitivity to iPR and ZR but not to respective CK-bases. On the other hand, overexpression of *AtENT8* confers hypersensitive reaction to iPR but not to iP. Moreover, experiments on *Arabidopsis* hypocotyls indicated that ^{3}H-iPR uptake efficiency is reduced by 40% in *AtENT8* and *AtENT3* mutants (Sun et al., 2005).

Hirose et al. (2008) performed a biochemical characterization of transport of CK-ribosides by AtENT3, 6 and 7 in yeast, showing that AtENT6 could possibly play a role in transport of iPR and tZR whereas AtENT3 and AtENT7 might contribute only weakly to the transport of CKs. In *Arabidopsis*, *AtENT6* expression was detected in root, leaf and flower vasculature (Hirose et al. 2008).

The rice genome turned out to contain 4 *ENTs* (designated *OsENT1-4*) (Hirose et al. 2005). However, only OsENT2 was able to complement the *ade2* (disabled adenine synthesis) yeast mutation on adenine-free minimal medium supplied with adenosine and was the only one to cause lethality of *fui1* (disabled uridine transport) yeast strain in the presence of toxic compound fluorouridine. Furthermore, competition studies showed that adenosine transport is partially inhibited by iPR. Indeed, subsequent direct measurement of ^{3}H-iPR and ^{3}H-ZR indicated higher affinity of OsENT2 for iPR than for ZR. As for the expression pattern of *OsENT2*, the β-glucuronidase gene expression driven by *OsENT2* promoter was detected in the scutellum during germination and in root and shoot vasculature of rice plants (Hirose et al. 2005).

4.6 Tobacco suspension-grown cells and cytokinins

Tobacco cultured cells were already shown to be a suitable model for revealing some aspects of CK physiology, namely transport, metabolism and cell cycle regulation.

4.6.1 Cytokinin uptake, excretion and metabolism

Experiments by Laloue et al. (1977) showed that the absorption rate of iP and iPR did not differ significantly in *Nicotiana tabacum* L. cv. Wisconsin 38 suspension cells and subsequent formation of mono-, di- and triphosphates was observed for both CKs. However, formation of iP 7-glucoside was recorded only in iP treated suspension cells. Also a fast degradation of iPR to adenylic products was recorded, which further increased 3-4 hours after exposing tobacco cells to iPR (Terrine and Laloue, 1980).

Excretion of CKs into the cultivation medium by *Nicotiana tabacum* L. cv. Virginia Bright Italia (VBI-0) cells in the course of the subcultivation interval was observed by Petrášek et al. (2002) and the dynamics of Z, iP, DHZ and their ribosides in the cultivation medium was proportional to the content of CKs in the cells. The ability of plant cultured cells to excrete endogenously produced CKs was shown also in *Nicotiana tabacum* L. cv. Wisconsin 38 suspension-grown cells: CK species detected in the cultivation medium included CK bases, CK ribosides and even CK nucleotides (Motyka et al., 2003).

4.6.2 Cell cycle dependent regulation of CKs content

In synchronized *Nicotiana tabacum* L. cv. Xanthi cells, tZ, tZR and an undefined CK-glucoside were detected and their content was found to peak together with the increase of mitotic index (Nishinari and Syōno, 1980). A more detailed analysis of 16 CK species in synchronized *Nicotiana tabacum* L. cv. Bright Yellow 2 (BY-2) cells confirmed Z- and DHZ-type CKs to increase sharply at the end of the S phase and during mitosis pointing to their possible role in the cell cycle progression of plant cells (Redig et al., 1996). Indeed, later study identified Z out of 7 tested CKs (tZ, tZR, DHZ, iP, iPR, BA, kinetin) to be able to override the inhibitory effect of CK biosynthesis inhibitor lovastatin in G2/M transition in BY-2 cells (Laureys et al., 1998). Despite the S-phase-linked increase in CK content mentioned above, G1/S cell cycle transition required only a low dose of CK; moreover, application of external Z prevented normal progression through the S-phase (Laureys et al., 1999).

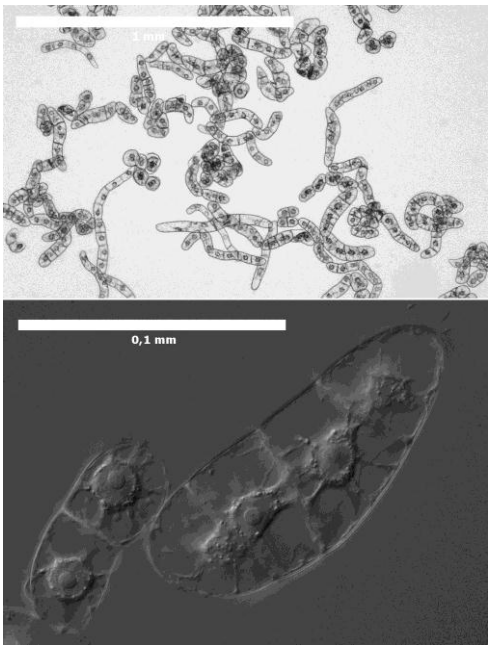

Figure 5 BY-2 cells in exponential growth phase.

Interestingly, changes in CK level were accompanied with oscillation of CKX activity in synchronized BY-2 cells. The maximum of CKX activity was recorded at the beginning of the S-phase and the minimum corresponded to the mitotic index culmination (Dobrev et al., 2002).

4.6.3 Cytokinins as apoptotic inducers

Contrary to commonly accepted cytokinesis-promoting nature of CKs micromolar doses of iP and BAR severely decreased viability of BY-2 cells. Exposition of BY-2 cells to 10-80 µM iP resulted in typical apoptotic-like changes including cell shrinkage, chromatin condensation and nuclear DNA degradation, as well as caspase-like proteases activation, altogether seriously marking cell growth rate (Mlejnek and Procházka 2002). At similar concentrations, BAR exhibited similar effects in BY-2 suspension cells, and main causes of its apoptotic effects were identified in depletion of intracellular ATP required for metabolic flow to BARMP and accompanying massive production of reactive oxygen species (ROS) (Mlejnek et al., 2003). The ATP depletion, but not reactive oxygen species formation, was subsequently recognized to be decisive for onset of apoptosis (Mlejnek et al., 2005).

5. Materials and methods

5.1 Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) or Duchefa Biochemie (Haarlem, Netherlands) unless otherwise stated. Unlabelled cytokinins were obtained from OlChemIm (Olomouc, Czech Republic). [2-^{3}H]iP (specific activity 1184 TBq×mol^{-1}), [2-^{3}H]iPR (1184 TBq×mol^{-1}), [2-^{3}H]*trans*Z (851 TBq×mol^{-1}), [2-^{3}H]*cis*Z (777 TBq×mol^{-1}), [2-^{3}H]*trans*ZR (851 TBq×mol^{-1}), [2-^{3}H]BA (1384 TBq×mol^{-1}) and [2-^{3}H]BAR (1517 TBq×mol^{-1}) were synthesized at the Isotope Laboratory, Institute of Experimental Botany AS CR, v. v. i. (Prague, Czech Republic) according to Hanuš et al. (2000). Cold cytokinins were dissolved in 1 N NaOH and dilution series (2500× concentrated stocks) was used in competition experiments in order to add equal amount of solvent to all variants. CCCP was dissolved in dimethyl sulfoxide (DMSO). Final concentration of DMSO was 0.05 %.

5.2 Plant material

Cells of the tobacco line BY-2, *Nicotiana tabacum* L. cv. Bright Yellow 2 (Nagata et al., 1992) were used for all the experiments and were cultivated in darkness at 27 °C on the orbital cultivator (IKA KS501, IKA Labortechnik, Staufen, Germany; 150 rpm) in liquid medium (3% [w/v] sucrose, 4.3 g×l^{-1} Murashige and Skoog salts, 100 mg×l^{-1} inositol, 1 mg×l^{-1} thiamin, 0.2 mg×l^{-1} 2,4-D, and 200 mg×l^{-1} KH_2PO_4, pH 5.8) and subcultured weekly (3 ml of suspension into 100 ml of fresh medium). Stock BY-2 calli were maintained on the same media solidified with 0.6% (w/v) agar and subcultured monthly. Cells in the exponential growth phase, 48 hours after subcultivation, were used for all experiments.

5.3 Cytokinin accumulation assays

Accumulation assays were performed according to Petrášek et al. (2003). Cytokinin accumulation was measured in BY-2 cells 48 hours after subcultivation in 0.25 ml or 0.5 ml (unless otherwise stated) aliquots of cell suspension (cell density approximatelly 7×10^5 cells×ml^{-1} was determined by counting in the Fuchs-Rosenthal hemocytometer).

Cultivation medium was removed by filtration on 20 µm mesh nylon filters and cells were resuspended in uptake buffer (20 mM MES, 10 mM sucrose, 0.5 mM $CaSO_4$, pH adjusted to 5.7 with KOH) and equilibrated for 45 minutes on the orbital shaker at 27°C in darkness. Equilibrated

cells were collected by filtration, resuspended in fresh uptake buffer and incubated with continuous orbital shaking for another 90 minutes under the same conditions. Radiolabelled CKs were added to the cell suspension to give a final concetration of 2 nM. At given time points (depending on the experiment), aliquots of cell suspension were sampled and accumulation of radiolabelled CK was terminated by rapid filtration under reduced pressure on cellulose filters (22 mm in diameter). Cell cakes with filters were transferred to scintilation vials, extracted with ethanol for 30 minutes and radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 2900TR scintillation counter, Packard Instrument Co., Meridien, CT, USA). Counts were corrected for surface radioactivity by subtracting counts of aliquots collected right after addition of radiolabelled CKs. Counting efficiency was determined by automatic external standardization and counts were corrected automatically. Inhibitors or competitors were added as required from stock solutions to give appropriate final concentration and proper controls were applied. All accumulation measurements were done at least in triplicates and SE (standard error of the mean) is shown. Recorded accumulation values are converted to the density of 1 million of cells per ml or are expressed as percentage of the control.

5.4 HPLC based metabolic profiling

48 hours after subcultivation, cells were prepared by equilibration in uptake buffer following the same protocol as described for the accumulation assay. Cells were incubated with 20 nM radiolabelled CKs for given time period (1, 5, 10 and 15 min). 200 mg FW of cells were removed by vacuum filtration and frozen in liquid nitrogen. Further extraction and purification of CK metabolites were adapted from method described by Dobrev and Kamínek (2002).

200 mg (FW) of BY-2 cells were placed in eppendorf tubes, 300 µl of cold modified Bieleski's solution was added (methanol/water/formic acid, 15:4:1 v/v/v) and cells were homogenized on RETSCH M301 (RETSCH, Haan, Germany) mixer mill at 30 Hz for 4 min. After addition of 1.2 ml of cold Bieleski solution and an overnight incubation at -20°C, samples were centrifuged for 10 minutes at 18000 rpm and 4°C (Jouan AM2.18; Jouan, Saint-Herblain, France) to separate solids. Supernatant was collected in falcon tubes and the pellet was resuspended in 1 ml of cold Bieleski's solution and re-extracted. Pooled supernatants were evaporated to achieve water fraction 0.2 ml or less. Residue was diluted in 5 ml of 1 M formic acid and purified on Oasis MCX mixed mode, cation-exchange, reverse-phase column (Waters, 6 cc/150 mg). The column was pre-conditionned with 5 ml of water and 5 ml of 1 M formic acid and the sample was applied on column. Subsequently, the column was washed with 5 ml of 1 M formic acid, followed by 5 ml of 1 M methanol to elute auxins and abscisic acid. Next, CK nucleotides were eluted with 5 ml of

0.35 M ammonium hydroxide followed by elution of CK bases, ribosides and glucosides with 5 ml of 0.35 M ammonium hydroxide in 60% methanol. After passing of each solvent the columns were purged briefly with air. Solvents were evaporated into dryness with a rotary vacuum evaporator at 40 °C.

Evaporated samples were dissolved in 50 µl of 15% (v/v) acetonitrile and used for the detection of [^{3}H]CK metabolites by high performance liquid chromatography (HPLC). HPLC consisted of series 200 autosampler and quaternary pump (both Perkin Elmer, USA), column Luna C18(2) (Phenomenex) heated at 35 °C and two detectors coupled in series: 235C diode array detector (Perkin Elmer, USA) and Ramona 2000 flow-through radioactivity detector (Raytest, Germany). Two solvents (A: 40 mM CH_3COOH adjusted with NH_4OH to pH 4.0 and B: CH_3CN/CH_3OH, 1/1, v/v) were used at flow rate of 0.6 ml min^{-1}. The column eluate was monitored at 270 nm by the diode array detector, and after on- line mixing with 3 volumes of liquid scintillation cocktail (Flo-Scint III, Packard BioSci- ence Co., Meriden, CT) it was monitored by a Ramona 2000 radioactivity detector. The metabolites of [^{3}H]CKs were identified on the basis of comparison of their retention times with authentic standards.

5.5 Determination of CKX activity

The cytokinin oxidase/dehydrogenase (CKX) from BY-2 cells was extracted and partially purified according to (Motyka et al. 2003) and its activity and substrate specificity were determined by *in vitro* radioisotope assays based on the conversion of [2-^{3}H]-labelled CKs ([2-^{3}H]iP, [2-^{3}H]tZ and [2-^{3}H]cZ) to [^{3}H]adenine. The assay mixture (50 µl final volume) included 100 mM TAPS-NaOH buffer containing 75 µM 2,6-dichloroindophenol (pH 8.5), 2 µM [2-^{3}H]CK (7.4 TBq mol^{-1} each), and enzyme preparation equivalent to 10-20 mg tissue FW (corresponding to 0.7 to 1.2 mg protein g^{-1} FW for BY-2 cells). After 4 hours incubation at 37 °C the reaction was terminated and the substrate was separated from the product of the enzyme reaction by HPLC, as described elsewhere (Gaudinová et al., 2005). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

6. Results

6.1 CKs are rapidly accumulated in BY-2 cells

To describe the early dynamics of CKs uptake in BY-2 cells, short 10 minutes accumulation experiments were performed with bioactive CK-bases, namely [^{3}H]*t*Z, [^{3}H]iP and [^{3}H]BA, and their respective ribosylated forms, [^{3}H]*t*ZR, [^{3}H]iPR and [^{3}H]BAR (**Fig. 6**). Each examined compound was tested in series of 3 independent accumulation runs. In this particular experiment collected data were not corrected for surface radioactivity. Drawback to this approach is that we do not eliminate the offset of the curve caused by rapid cell wall contamination. A minor spread between the recorded values of the three independently run variants supports the validity of the experimental model.

6.1.1 Accumulation of CK-bases

CK-bases are readily taken up by tobacco BY-2 cells (**Fig. 6**). There was a steady increase in their accumulation which started immediately after addition of radiolabelled CKs to reach 2 nM concentration. It should be noted that the first sampling took place not earlier than approximately 10 seconds after addition of the tritiated substrate which is the limit of the experimental setup. Interestingly, a possible cell wall contamination effect was recorded in case of BA uptake where the offset of the accumulation curve was the strongest one.

The accumulation appears to be non-linear in case of tZ and iP, whereas linear dependency is apparent in case of BA after the initial rapid increase. The limited experimental runtime did not reveal further development of the curve. Checking the shape of control accumulation curves in a longer run (**Fig. 10**) showed the same tendencies: non-linearity of tZ and iP, but not of BA uptake.

6.1.2 Accumulation of CK ribosides

The accumulation of CK-ribosides follows a pattern similar to CK-bases, i. e. a stable increase in accumulation of externally added tZR, iPR and BAR during the 10 minutes experimental timeframe (**Fig. 6**). The transport of tZR and iPR appears limitless within this time span. However, a brief look at longer accumulation assays (**Fig. 10**) reveals that uptake rate of iPR and BAR but not ZR may reach a limit within a larger timescale experiment. To elucidate the possible saturability of CKs transport, I performed a set of additional competition experiments presented further in this thesis.

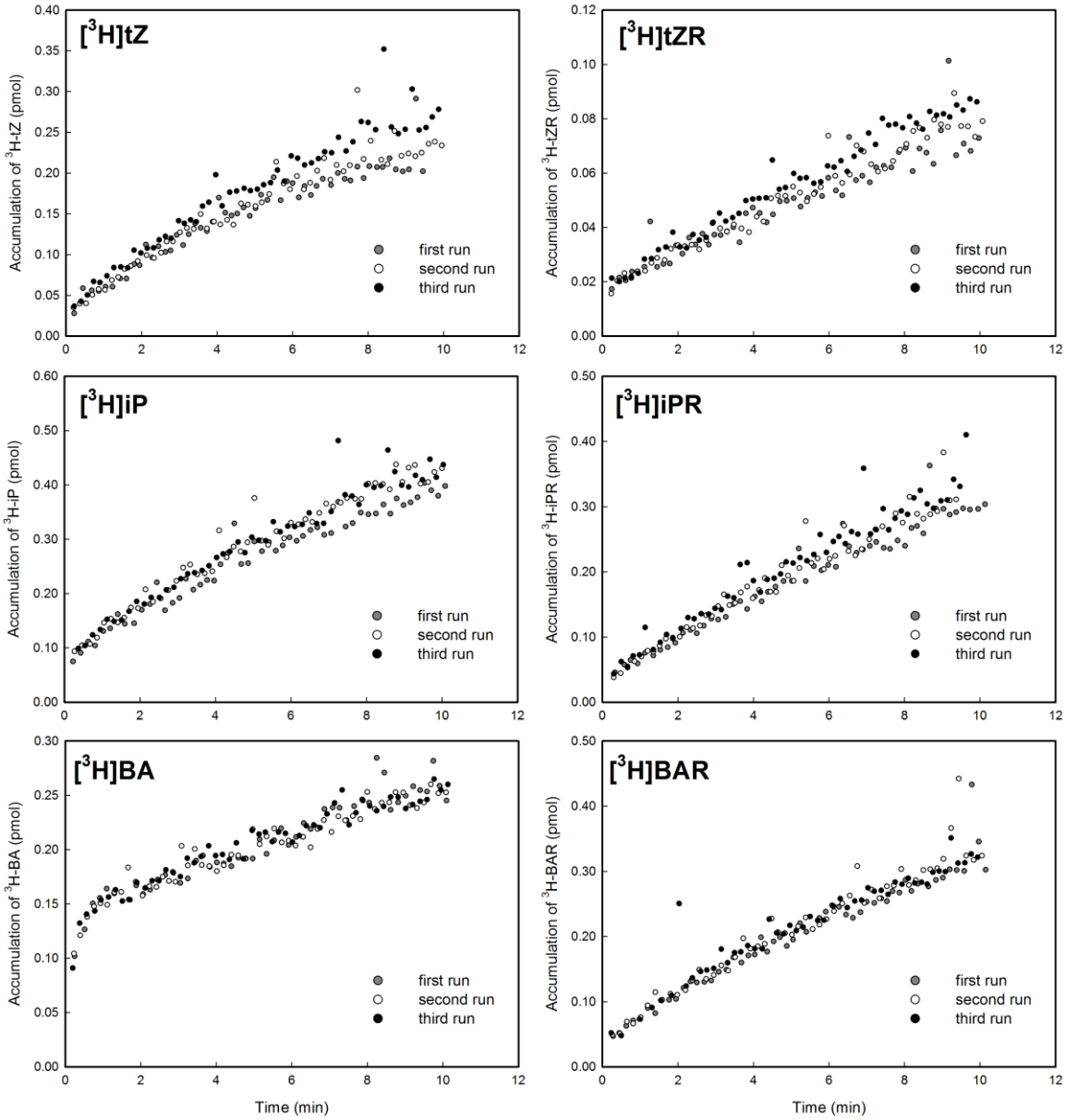

Figure 6 Accumulation of [^{3}H]CKs in BY-2 cells. **[LEFT]** Accumulation of CK-bases (tZ, iP and BA). **[RIGHT]** Accumulation of CK-ribosides (tZR, iPR and BAR). Concetration of tested CKs is 2 nM. x-axis displays time in minutes, y-axis displays the accumulation of respective [^{3}H]CK in pmols. 0.5 ml samples.

6.1.3 *Cis*-zeatin and *trans*-zeatin uptake do not differ

Accumulation rates of cZ and tZ in BY-2 suspension cells were examined in order to elucidate a possible role of high portions of *c*Z in certain plant species. A significant difference in uptake may correspond to a difference in the ability of triggering signalling events (**Fig. 7**). However, there was no particular difference in accumulation pattern of both zeatin isomers. During a 40 min long experiment, *cis*Z accumulation differred neither from that of tZ (**Fig. 7**), nor iP (see **Fig. 8**, for an example). These results were published in paper which is part of this thesis (Gajdošová et al., 2011).

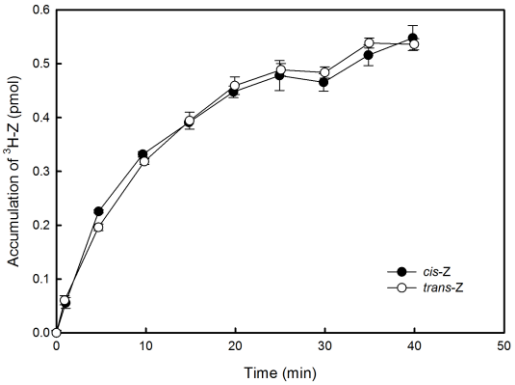

Figure 7 Comparison of *cis*-zeatin and *trans*-zeatin uptake in BY-2 cells. Concetration of tested CKs is 2 nM. x-axis displays time in minutes, y-axis displays the accumulation in pmols of respective [^{3}H]zeatin isomer in 0.5 ml suspension samples. Error bars show SEM (n=4).

6.2 Competition between radiolabelled and cold CKs

Competition studies represent a suitable tool for determination of kinetic parameters of enzyme-substrate interaction. The relation between the transporter and the transported solute may be regarded as an analogy to enzyme ligand-binding studies. Therefore the same methodology and terminology is often applied. From this point of view, a competition experiment is a displacement assay performed by either the same chemical species (homologous displacement) or by a different chemical species (heterologous displacement) of the competitor (Rovati, 1998). I did series of homologous and heterologous displacements studies to test the properties of putative transmembrane CK transporters. The main purpose was to test the hypothesis of two distinct transport routes of CK-bases and CK-ribosides that would correspond to the suggested cytokinin transport activities of purine permeases and equilibrative nucleoside transpoters.

6.2.1 CK transport saturates at elevated concentrations of cold competitor of the same species

Competition of [^{3}H]tZ, [^{3}H]iP, [^{3}H]tZR and [^{3}H]iPR with the cold competitor of the same species applied in increasing concentration of the cold competitor of the same species reveals a gradually decreasing accumulation of the radiolabelled tracer in all cases (**Fig. 8**). With the exception of iP, it is obvious that effective competition is reached with competitor applied at concetrations higher than 20 nmol×l^{-1}. iP competition is reached already with the smallest, 2 nM, concentration of the cold competitor and competition further gradually increases gradually with its concentration.

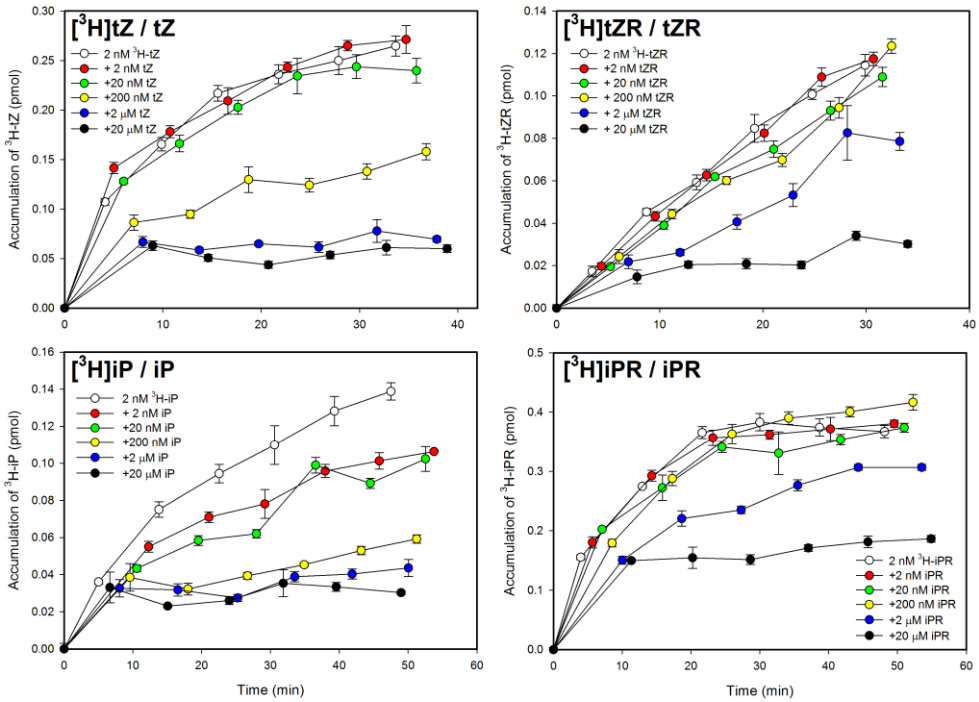

Figure 8 (LEFT) Competition between [^{3}H]-labelled and cold CK-bases (tZ or iP) **(RIGHT)** Competition between [^{3}H]-labelled and cold CK-ribosides (tZR or iPR). After mixing the cells with [^{3}H]CK, a sample was removed for correction of cell surface contamination, the cell suspension was divided into aliquots and competitors (2 nM, 20 nM, 200 nM, 2 µM, 20 µM) were added before proceeding. X-axis displays time in minutes, y-axis displays the accumulation of respective [^{3}H]CK in pmols. iPR: 0.5 ml samples. Error bars show SEM (n=4).

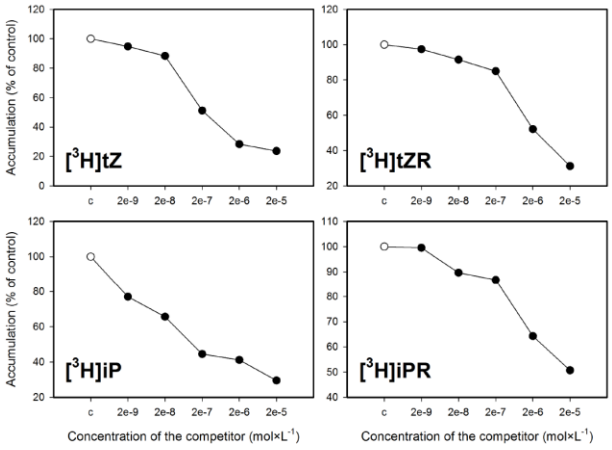

Figure 9 Displacement curves of tZ, tZR, iP and iPR. Control (c; open circles) concentration of the 'hot' substrate is 2 nM. Concentration of the competing 'cold' substrates (closed circles) is 2 nM, 20 nM, 200 nM, 2 µM and 20 µM respectively. Data for the curves are taken from graphs in Fig. 7 at 15 min interval.

Figure 9 depicts the actual displacement curves constructed with data taken from **Figure 8** graphs at 15 min timepoint. The displacement constant K_d which indicates half the capacity of the putative transporters for translocation of a given CK corresponds to the inflexion point of the curve. Actually, in this case the prediction is only indicative as the concentration range required for proper reading was probably not fully covered. However, the most precise reading of K_d value for tZ may be close to 100 nM, and a relatively reasonable prediction of K_d value for tZR and iPR would actually be 1 µM or higher. Based on the data obtained, no reliable estimation is possible to make for iP.

6.2.2 CK-bases and CK-ribosides show limited uptake competition

In order to test the interdependence of CK-bases and CK-ribosides transport in BY-2 cells, displacement experiments between radiolabelled tracers and 'cold' competitors of the different, complementary species were performed. In other words, [^{3}H]tZ was subjected to a competition by cold tZR, [^{3}H]iP was competed by cold iPR and vice versa (**Fig. 10**). All other experimental conditions remained the same as in the previous "same species" displacement assays.

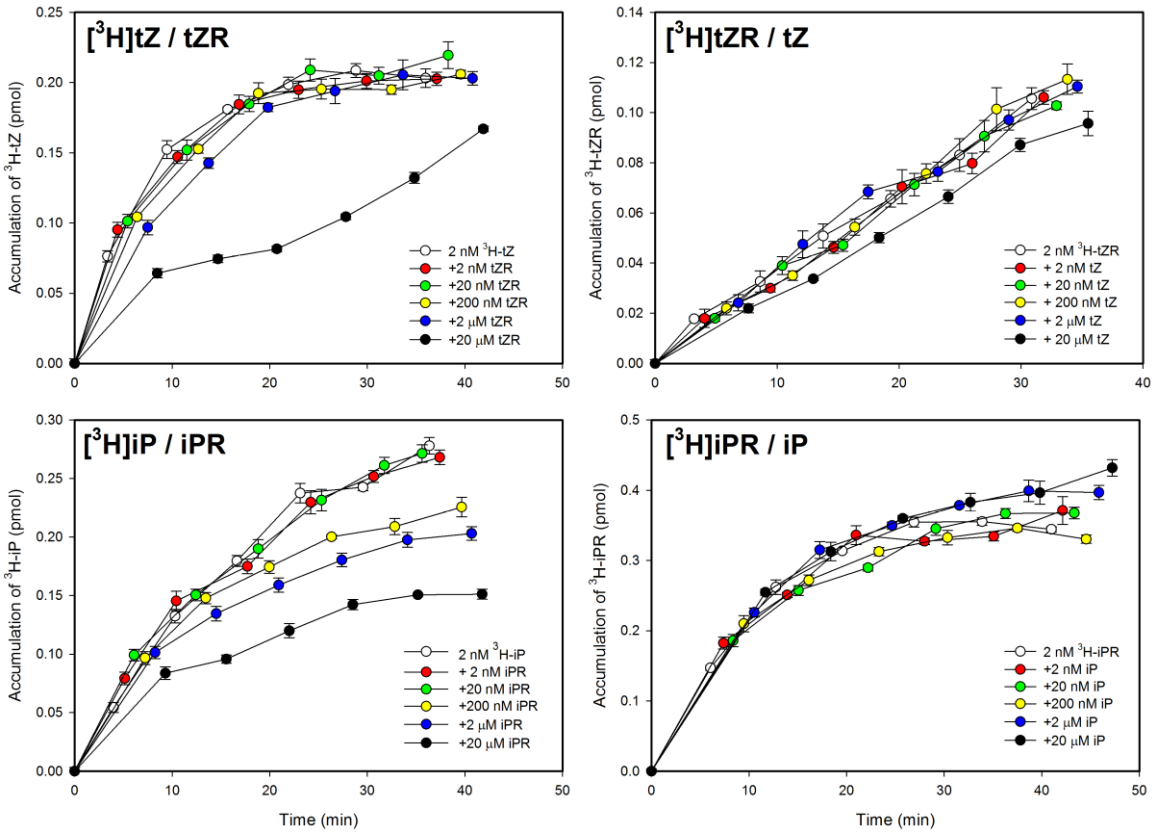

Figure 10 (LEFT) Competition between [^{3}H]CK-bases (tZ or iP) and a corresponding cold CK-riboside (tZR and iPR, respectively). **(RIGHT)**. Competition between [^{3}H]CK-riboside (tZR or iPR) and a corresponding cold CK-base (tZ and iP, respectively). After mixing the cells with [^{3}H]CKs, a sample was removed for correction of cell surface contamination, the cell suspension was divided into aliquots and competitors (2 nM, 20 nM, 200 nM, 2 µM, 20 µM) were added before proceeding. X-axis displays time in minutes, y-axis displays the accumulation of respective [^{3}H]CK in pmols. iPR: 0.5 ml samples. Error bars show SEM (n=4).

From the collected data it is obvious that uptake competition between the two cytokinin forms is limited. tZ accumulation is not perturbed by tZR up to 2 µM tZR concentration, and massive surplus of the competitor (20 µM) is necessary to alter the accumulation curve. iP accumulation is interfered by iPR at concentration of 200 nM and higher. Reciprocally, tZR and iPR do not show any sign of accumulation change uder the influence of CK-base competitors at any tested concentration.

6.2.3 Displacement testing with other CKs, adenine and adenosine

To further test the potential competition of CK-bases and CK-ribosides for the same transport route, I used a broader spectrum of CKs and other related substances like adenine and adenosine and set up competition experiments proceeded under same conditions but using only one concetration of cold competitors, 20 µM, as it turned out to be the one reliably efficient in previous attempts (**Fig. 11**).

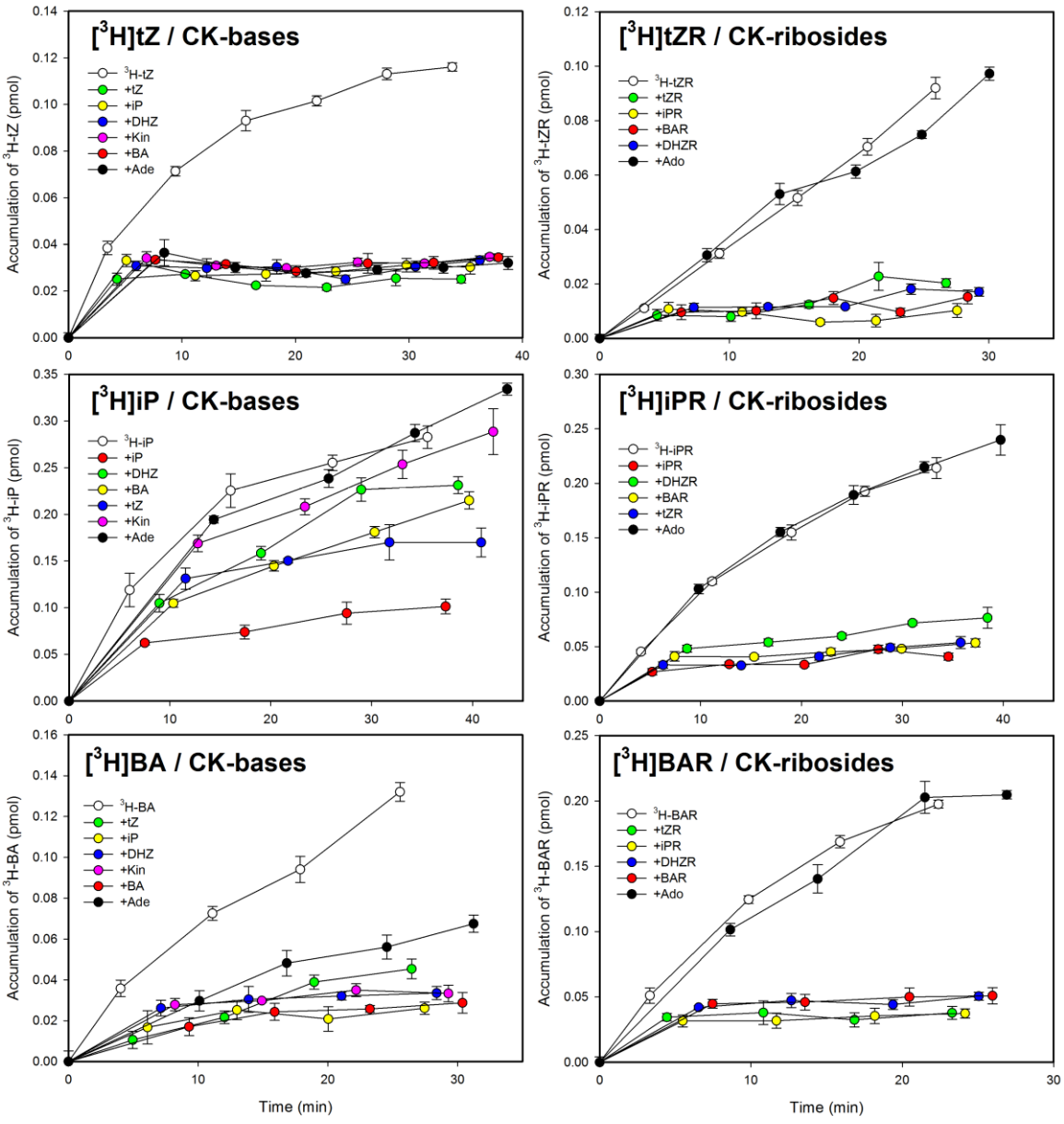

Figure 11 (LEFT) Competition between 2 nM [^{3}H]CK-bases (tZ, iP, BA) and cold CK-bases (20 µM). **(RIGHT)** Competition between 2 nM [^{3}H]CK-ribosides (tZR, iPR, BAR) and cold CK-ribosides (20 µM). After mixing the cells with [^{3}H]CKs, a sample was removed for correction of cell surface contamination, the cell suspension was divided into aliquots and competitors were added before proceeding. X-axis displays the time in minutes, y-axis displays the accumulation of respective [^{3}H]CK in pmols. iP and iPR: 0.5 ml samples. Error bars show SEM (n=4).

Evidently, competition takes place between the same species of competitors (CK-base vs. CK-bases, CK-riboside vs. CK-ribosides) in most cases. Exceptionally, iP accumulation is not perturbed to a similar extent by a high concentration excess of CK-bases competitors. Notably, adenosine is the only substance that does not disturb the accumulation rate of any of the 3 tested CK ribosides.

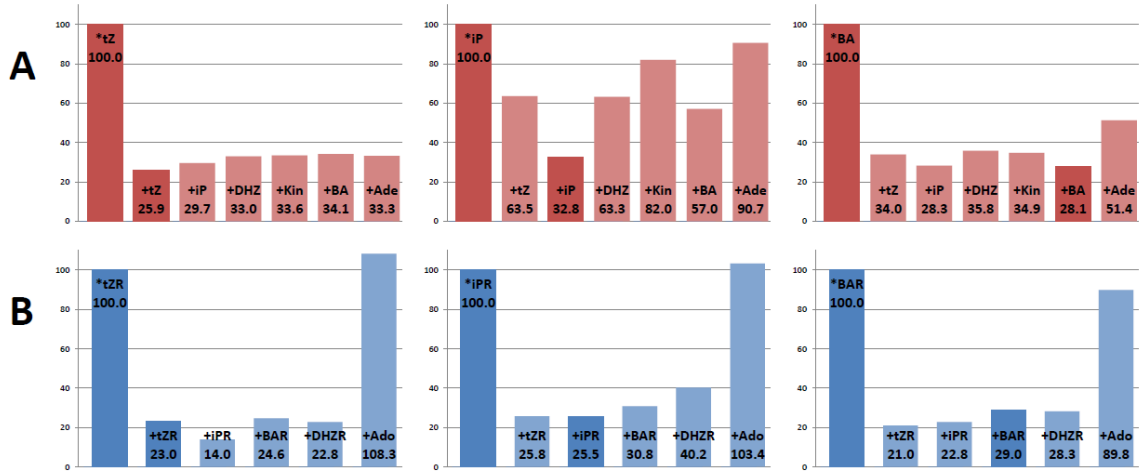

Figure 12 (A) Competition between [^{3}H]CK-bases (2 nM) and cold CK-bases (20 µM). **(B)** Competition between [^{3}HCK-ribosides (2 nM) and cold CK-ribosides (20µM). Accumulation values of [^{3}H]CK (*tZ, *iP, *BA, *tZR, *iPR, *BAR) equal 100 %. Competitors were added at the beginning of the experiment and a decrease of accumulation of [^{3}H]CKs is expressed as percentage of control. Saturated colors represent competition by the same CK-species. Data were taken at 15 min interval from Fig. 11.

A review of accumulation decrease 15 minutes after the beginning of measurement expressed as percentage of control value is presented in **Fig. 12**. If an effective competition occurs, the accumulation of the radiolabelled tracer usually falls down to approximatelly 30 % compared to control. A good measure to this claim may be the control represented by the accumulation ratio of the cold competitor of the same species, marked always with the same saturated color in the figures. Interestingly, the uptake specificity turns out to be the most variated in case of iP, ranging from 60 % level for competition by tZ, DHZ and BA, to 80 or 90 % levels for competition by kinetin and adenine, respectively. Also, adenine decreases the accumulation of [^{3}H]BA to 50 % level. Adenosine presence, as mentioned above, seemingly does not significantly affect the uptake rates of tested CK-ribosides.

Competition experiments between [^{3}H]tZ or [^{3}H]BA and cold CK-ribosides, and [^{3}H]tZR or [^{3}H]BAR and cold CK-bases yield several interesting results (**Fig. 13**). First, tZ and BA uptake is most efficiently supressed by tZR, adenosine and iPR. [^{3}H]tZ accumulation decreases to 30 % (tZR competition), 33 % (adenosine competition) and 57 % (iPR competition), and [^{3}H]BA

accumulation decreases to 33 % (tZR competition), 36 % (adenosine competition) and 48 % (iPR competition) (**Fig. 14**).

Maximal decrease caused by DHZR or BAR is by 17 % or less of the control accumulation. Compared to the results of displacement of [^{3}H]CK bases by cold CK ribosides, [^{3}H]tZR and [^{3}H]BAR accumulation is affected much less by competition with CK bases (**Figs. 13, 14**). The most prominent competitors at 15 minutes time interval picked from **Fig. 13** are iP and BA, both lowering the accumulation to 60-80 % of the controls. DHZ and kinetin compete tZR uptake by approximately 20 % efficiency and even less in the case of BAR uptake. Notable is the lack of inhibition of uptake of both tested CK ribosides by adenine which contrasts with a strong adenosine inhibition of CK bases accumulation (**Fig. 14**).

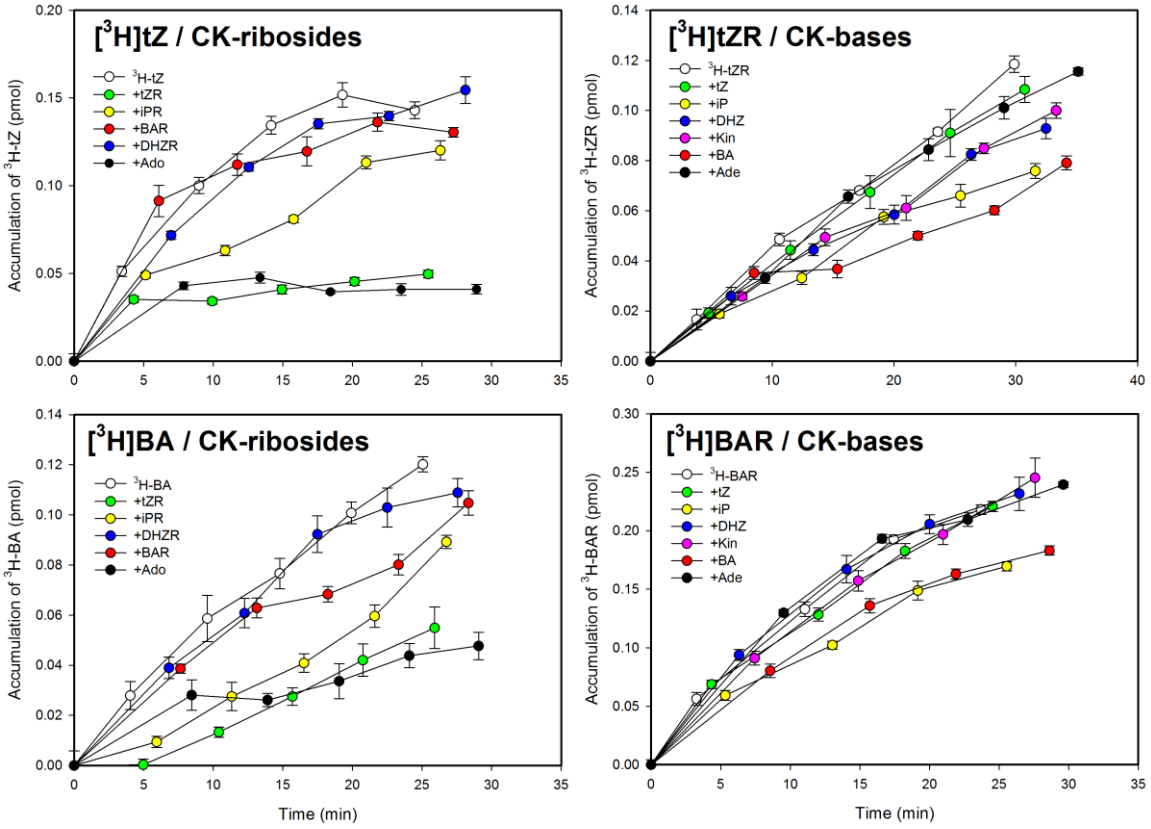

Figure 13 (LEFT) Competition between 2 nM [^{3}H]CK-bases (tZ, BA) and cold CK-ribosides (20 µM). **(RIGHT)** Competition between 2 nM [^{3}H]CK-ribosides (tZR, BAR) and cold CK-bases (20 µM). Competitors were added at the beginning of the experiment. X-axis displays the time in minutes, y-axis displays the accumulation of respective [^{3}H]CK in pmols. Error bars show SEM (n=4).

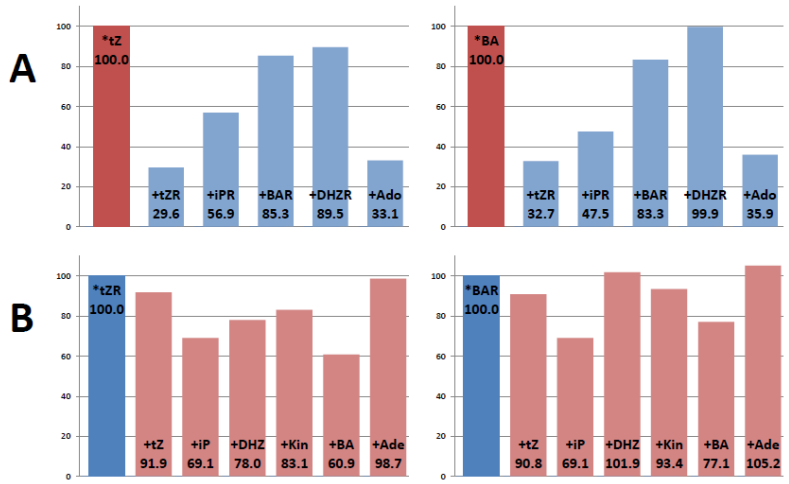

Figure 14 (A) Competition between [^{3}H]CK-bases (2 nM) and cold CK-ribosides (20 µM). **(B)** Competition between [^{3}H]CK-ribosides (2 nM) and cold CK-bases (20µM). Accumulation values of [^{3}H]CKs (*tZ, *BA, *tZR, *BAR) equal 100 %. Competition is expressed as a percentual decrease compared to control (saturated colors). Data were taken at 15 min interval from Fig. 13.

6.3 Uptake of cytokinins is sensitive to protonophore CCCP

Carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) is an uncoupling chemical agent used to disturb the H^+ equilibrium of lipid bilayers, thus affecting membrane processes depending on proton motive force, for example energy dependent solute transport or ATP synthesis.

CCCP affects the accumulation of both CK bases (tZ and iP) and CK ribosides (tZR and iPR) in BY-2 cells (**Fig. 15**). At the concentration of 50 µmol × L^{-1}, an "in flight" addition of CCCP practically abolishes further CK uptake, indicating that CKs translocation is energy dependent. The onset of CCCP effect is accompanied by approximately 1 minute delay, so addition of the inhibitor right after the beginning of the experiment mimicks the "in flight" variant, retaining the levels of the already taken up substrate. Notable is the steady state tendency of CCCP-influenced curves, which in accumulation terms means that there is no uptake and, equally, no excretion.

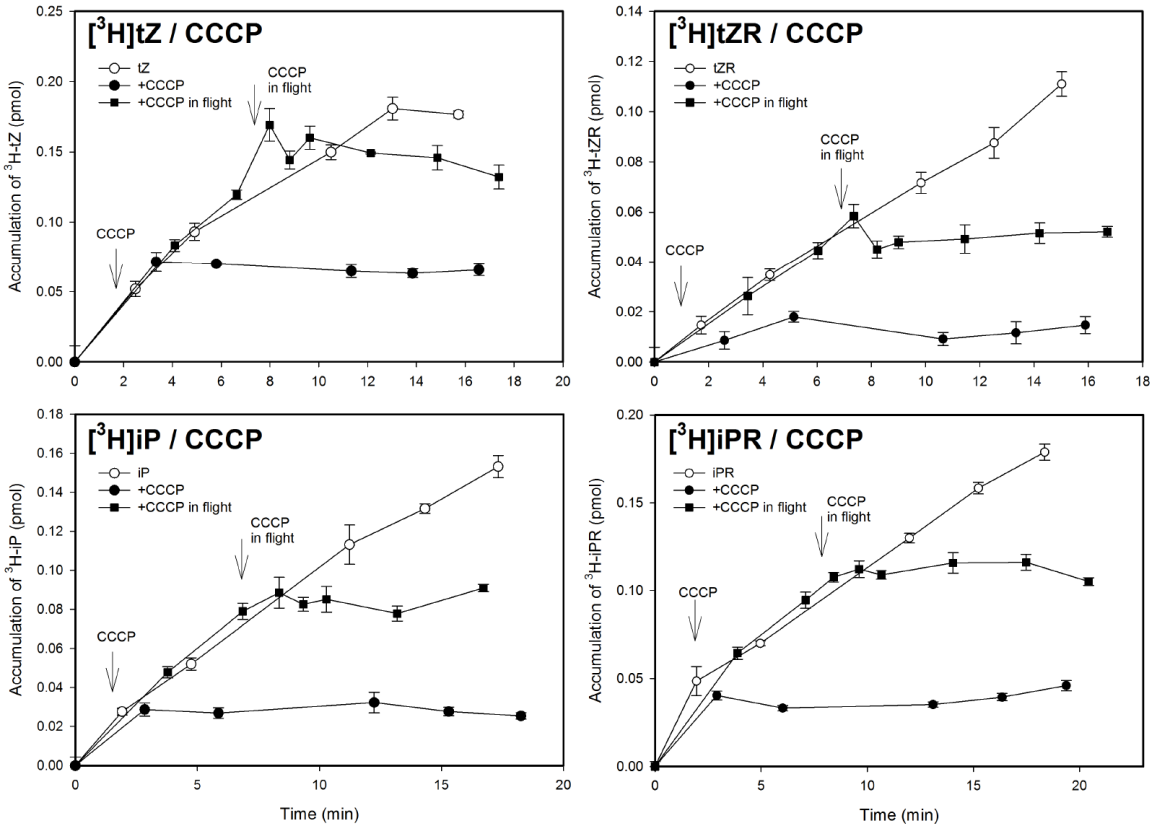

Figure 15 Energy dependence of CKs transport. Effect of carbonylcyanide *m*-chlorophenylhydrazone (CCCP, 50 µM) on accumulation of 2 nM **(LEFT)** CK bases (tZ and iP) and **(RIGHT)** CK ribosides (tZR and iPR). The first addition of CCCP was done immediately after the beginning of the experiment, the "in flight" addition then in the approximatelly seventh minute. CCCP addition times are marked with arrows. Error bars show SEM (n=4).

6.4 CKs are promptly metabolised upon entering BY-2 cells

To address the intracellullar fate of CKs accumulated in BY-2 cells, metabolic profiles of tritium labelled CK bases (tZ, iP and BA), and their respective ribosides (**Tab. 1**) were investigated (**Figures 17 and 18**). Samples were collected along the accumulation experiment in the first, fifth, tenth and fifteenth minutes, CK metabolites were extracted and subsequently analysed by HPLC connected in tandem with radioactivity detector. Any reference to the "first minute" sample means that the sample was removed as quickly as possible, usually within 20 seconds, after addition of the tritiated substrate. Due to required resolution of the chromatograms, accumulations were performed with 20 nM [^{3}H]CK concentration and 200 mg FW samples.

6.4.1 CK bases metabolic profiling

Overlays of chromatograms representing the four timepoints for all the three CK bases are shown in **Fig. 17**. An increase in intracellular accumulation of metabolites may be seen best distinguished among the extremities marked with red line corresponding to the first minute sample and black line corresponding to the fifteenth minute sample. Curiously, even in the first samples, lower than expected activities are present in peaks representing the actually accumulated substance. Instead, a shift of the radio label to the metabolites fraction is recorded from the very first minute. Just for the record, tritium is part of the purine ring, which allows identification of metabolic changes on the side chain. A time dependent proportional plot with a percentually represented metabolite distribution is shown in **Fig. 19**.

The purity of stock [^{3}H]CKs was examined by HPLC as well and is given in **Table 1**.

[^{3}H]CK	tZ	cZ	iP	BA	tZR	iPR	BAR
Purity [%]	86.2	90.4	91.0	89.7	94.3	91.8	83.5

Table 1 Purity of the stock solutions of CKs tested in the feeding experiment. Numbers represent the percentual portion of the total recorded radioactivity as determined by HPLC and identification of the resulting peaks on the basis of coincidence of their retention times with authentic standards.

A quick look at **Fig. 17** reveals two prominent metabolic conversions that are preferentially used for a fast inactivation of bioactive CK bases in BY-2 cells. First is the degradation of iP to adenine and the second is phosphoribosyl transfer to *trans*-zeatin and benzyladenine to form an appropriate CK-riboside monophosphate.

In terms of percents of total accumulated tracer, 91.5 % of the original stock tZ results in 46 % of total radioactivity just after entering the cell, decreasing further to 38 % after 5 minutes, 22 %

after 10 minutes and 14 % after 15 minutes. In the meantime, the portion of tZRMP, the main metabolite, increases respectively from 30 %, to 43 %, 53 % and finally to 58 % of total recorded radioactivity. Another main metabolite, tZR grows from 6 % to 16 % of total. Detected adenine and cZ levels are relatively stable around 5 % and 1 % share, respectively (**Fig. 19**).

iP is, from the starting stock purity of 93.4 %, readily degraded to adenine decreasing its portion to 41 % in the first sample followed by shrinking to 25 %, 14 %, and finally down to 10 % of total representation. The aforementioned degradation product, adenine, grows meanwhile from 44 %, through 56 %, to 67 % and 71 % of total detected activity. Other detectable metabolites are iPRMP levelling between a steady state 5-7 % and iP7G (isopentenyladenine 7-glucoside), which grows from 2 % to 4 % during the 15 minutes long experiment, and Ado, extending from under 1 % to aproximately 3 % (**Fig. 19**).

The third tested CK base, BA, enjoys 91 % initial radio-purity which in the cells diminishes to 73 % in the first recording and subsequently to 57 %, 42 % and finally 39 % of total sample radioactivity, while its monophosphate metabolite part evolves from the starting 13 % level into 46 % proportion after a quarter hour. Increased fractions of BA7G (benzyladenine 7-glucoside) and BA9G (benzyladenine 9-glucoside) are recorded as well, finishing at 6 % and 1.4 % respectively (**Fig. 19**).

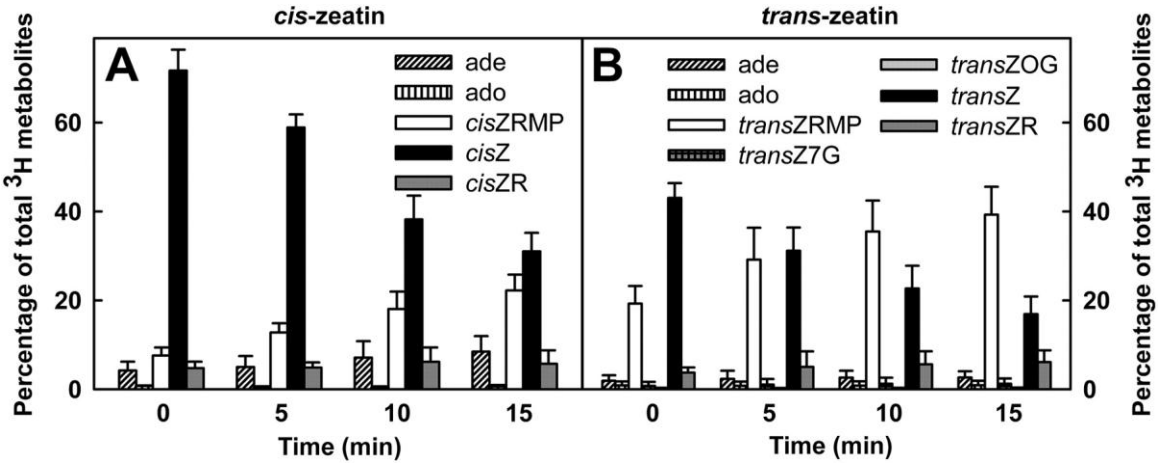

Figure 16 Metabolism of [^{3}H]*cis*Z and [^{3}H]*trans*Z in tobacco BY-2 suspension-cultured cells. The bars represent the distribution of radioactivity associated with individual CK metabolites 5, 10, and 15 min after application of [^{3}H]*cis*Z (A) or [^{3}H]*trans*Z (B); the values are expressed as a percentage of the total extracted radioactivity taken up by cells. Error bars represent the SD (Ade, adenine; Ado, adenosine; *cis*Z, *cis*-zeatin; *cis*ZR, *cis*-zeatin riboside; *cis*ZRMP, *cis*-zeatin riboside-5'-monophosphate; *trans*Z, *trans*-zeatin; *trans*ZR, *trans*-zeatin riboside; *trans*ZRMP, *trans*-zeatin riboside-5'-monophosphate; *trans*Z7G, *trans*-zeatin 7-glucoside; *trans*-ZOG, *trans*-zeatin O-glucoside). (Gajdošová et al., 2011)

An additional independent comparison of the short term metabolism of tZ and cZ was performed. cZ gradually decreased from 90 % of initial purity to 72 % ratio in the first sample and finished with 31 % portion of total accumulated radioactivity. After 15 minutes, the most

abundant metabolite was cZRMP (25 %), followed by adenine (15 %) and cZR (11 %). Compared to tZ, the metabolic changes of cZ were slower and yielded different spectrum of metabolites. Surprisingly, no *cis-trans* isomeration occured during the feeding experiment (**Fig. 16**). These results were published in paper which is part of this thesis (Gajdošová et al., 2011).

6.4.2 CK ribosides metabolic profiles

Similar chromatograms resulting from HPLC analysis of tZR, iPR and BAR are presented in **Figure 18**. For a percentual plot see **Figure 19**. The main way to quickly cope with excessive uptake of bioactive CK ribosides in BY-2 cells seems to be the phosphorylation to form the appropriate CK monophosphate. However, certain differences exist between tested CK ribosides.

tZR of stock solution purity higher than 96 % is immediately metabolised to ZRMP and preserves a share of 25 % on the first reading down to 20 % on the last, 15 minutes reading. At the expense of tZR, the tZRMP proportion grows from 59 % to 70 %. A small increment was seen for tZ which turned from 3 % to 4.5 % during the 15 minutes timeframe.

iPR of initial purity approximately 93 % drops down to 30 % ratio upon entering BY-2 cells and further decreases to 12 % share after a quarter hour run. The main metabolite, iPRMP reaches its peak ratio in the 5th minute, transitionning from 55 % of the first minute's relative abundance, through 64 %, 62 % to a final decrease to 55 %. This is probably due to an increase of Ado and Ade ratio from a mere 3 % for both substances up to 15 % in case of Ado and 12 % in case of Ade.

Last, BAR enters the cells with 86 % purity before being converted promptly to monophosphate. Also, BAR is the only CK tested whose intracellular metabolic ratio doesn't only decrease during the experiment but grows after the initial dropdown, with fractions evolving from 28 % in the first minute through 26 % on the second reading, 27 % after 10 minutes and a final increase to 30 % of relative abundance. Also, Ade and Ado minor ratios of total radioactivity are diminishing gradually.

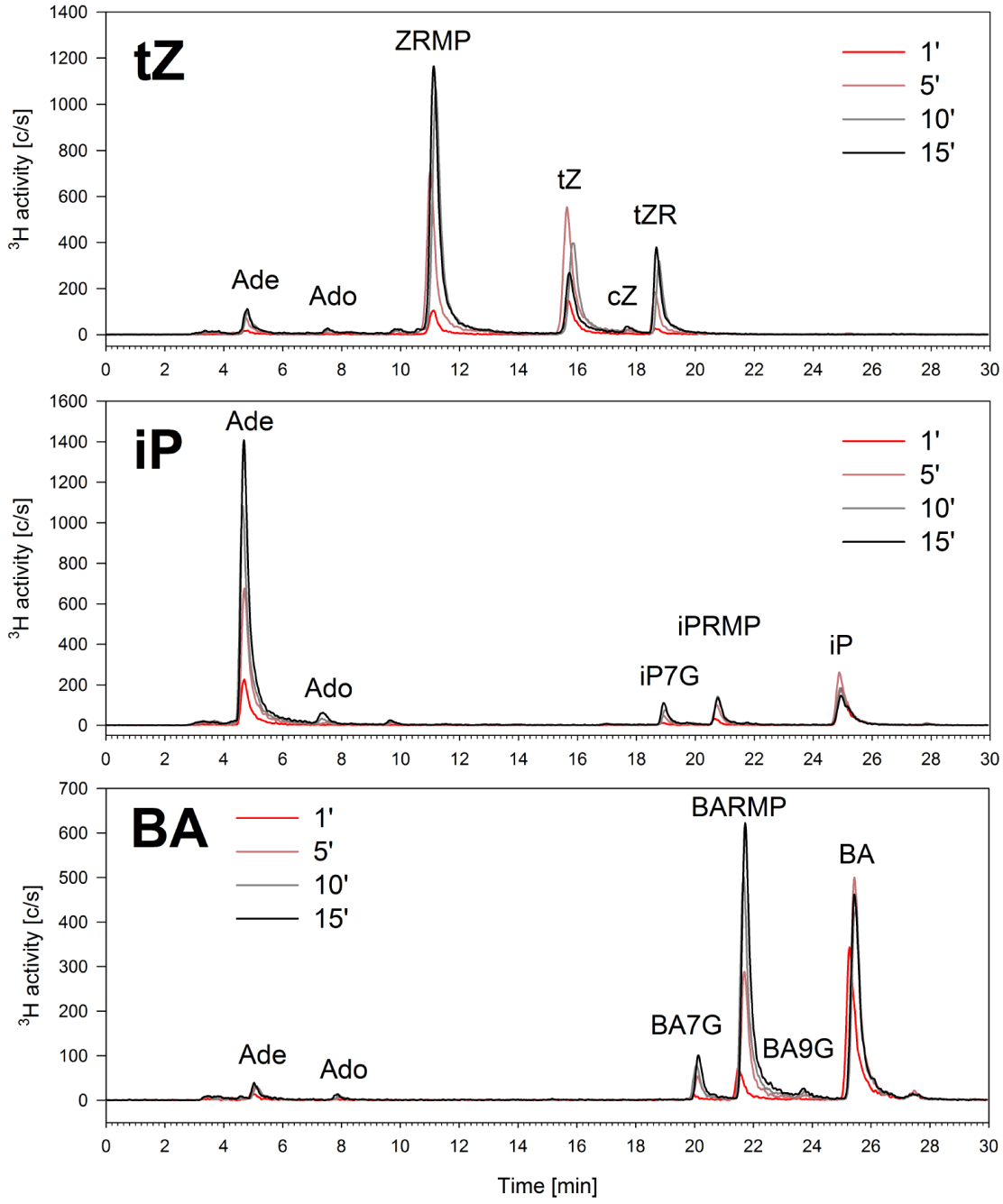

Figure 17 The HPLC chromatograms of metabolites of tested CK-bases. 48 hours after subcultivation, BY-2 cells were incubated with [^{3}H]tZ, [^{3}H]iP and [^{3}H]BA, and samples were removed in the first, fifth, tenth and fifteenth minute after beginning of the feeding experiment. Each graph represents overlay of HPLC chromatograms obtained by analysis of the 4 timepoint sample series. The metabolites were identified on the on the basis of coincidence of their retention times with authentic standards. Ade (adenine), Ado (adenosine), tZ (*trans*-zeatin), cZ (*cis*-zeatin), iP (isopentenyladenine), BA (benzyladenine), tZR (*trans*-zeatin riboside), ZRMP (*trans*-zeatin riboside monophosphate), iPRMP (isopentenyladenosine monophosphate), BARMP (benzyladenosine monophosphate), iP7G (isopentenyl 7-glucoside), BA7G (benzyladenine 7-glucoside), BA9G (benzyladenine 9-glucoside). X-axis represents time in minutes, y-axis represents ^{3}H activity in counts per second.

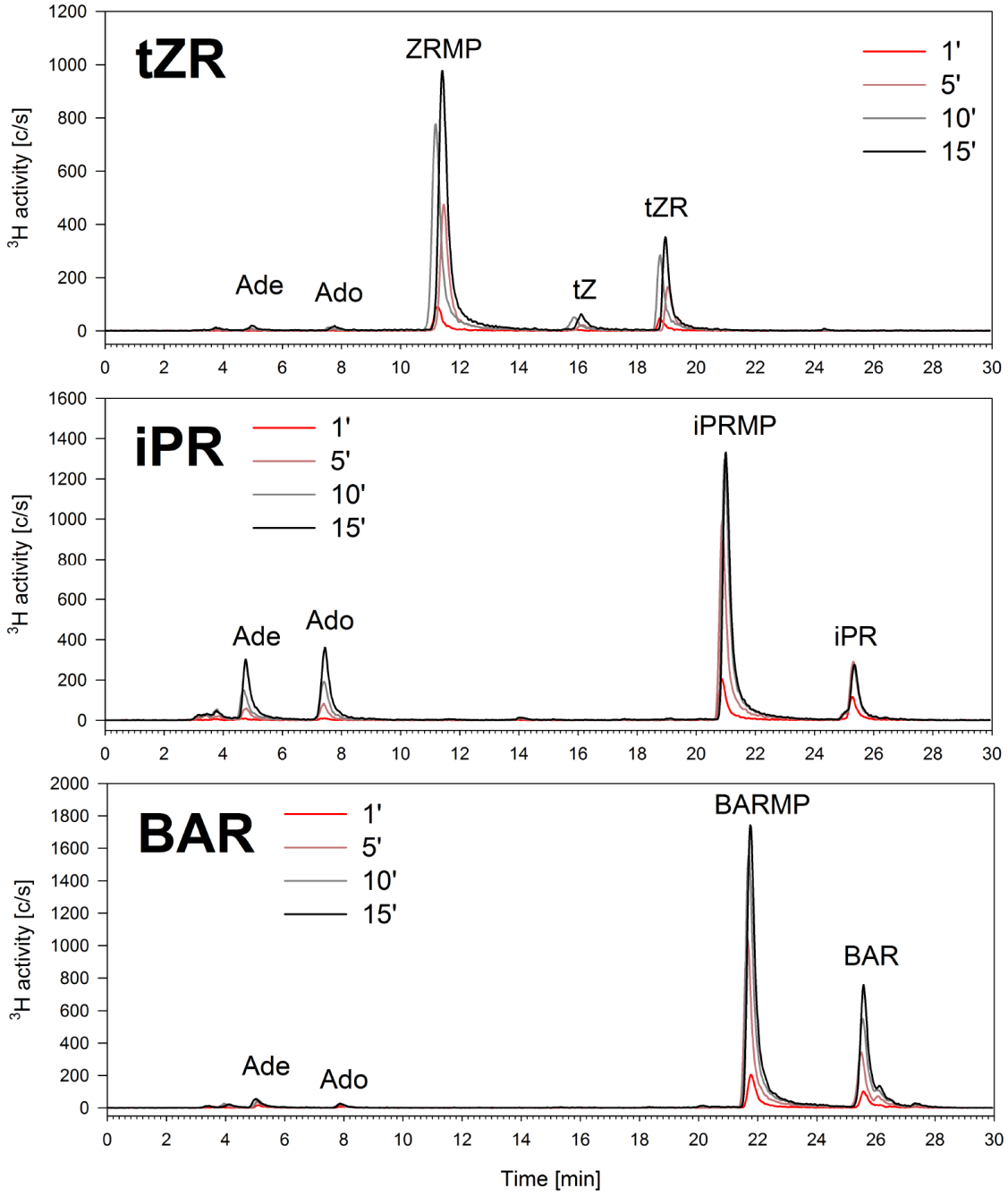

Figure 18 The HPLC chromatograms of metabolites of tested CK-ribosides. 48 hours after subcultivation, BY-2 cells were incubated with ^{3}H-tZR, ^{3}H-iPR and ^{3}H-BAR, and samples were removed in the first, fifth, tenth and fifteenth minute after beginning of the feeding experiment. Each graph represents the HPLC chromatograms obtained by analysis of the 4 timepoint sample series. The metabolites were identified on the on the basis of coincidence of their retention times with authentic standards. Ade (adenine), Ado (adenosine), tZ (*trans*-zeatin), tZR (*trans*-zeatin riboside), iPR (isopentenyladenosine), BAR (benzyladenosine), ZRMP (*trans*-zeatin riboside monophosphate), iPRMP (isopentenyladenosine monophosphate), BARMP (benzyladenosine monophosphate). X-axis represents time in minutes, y-axis represents ^{3}H activity in counts per second.

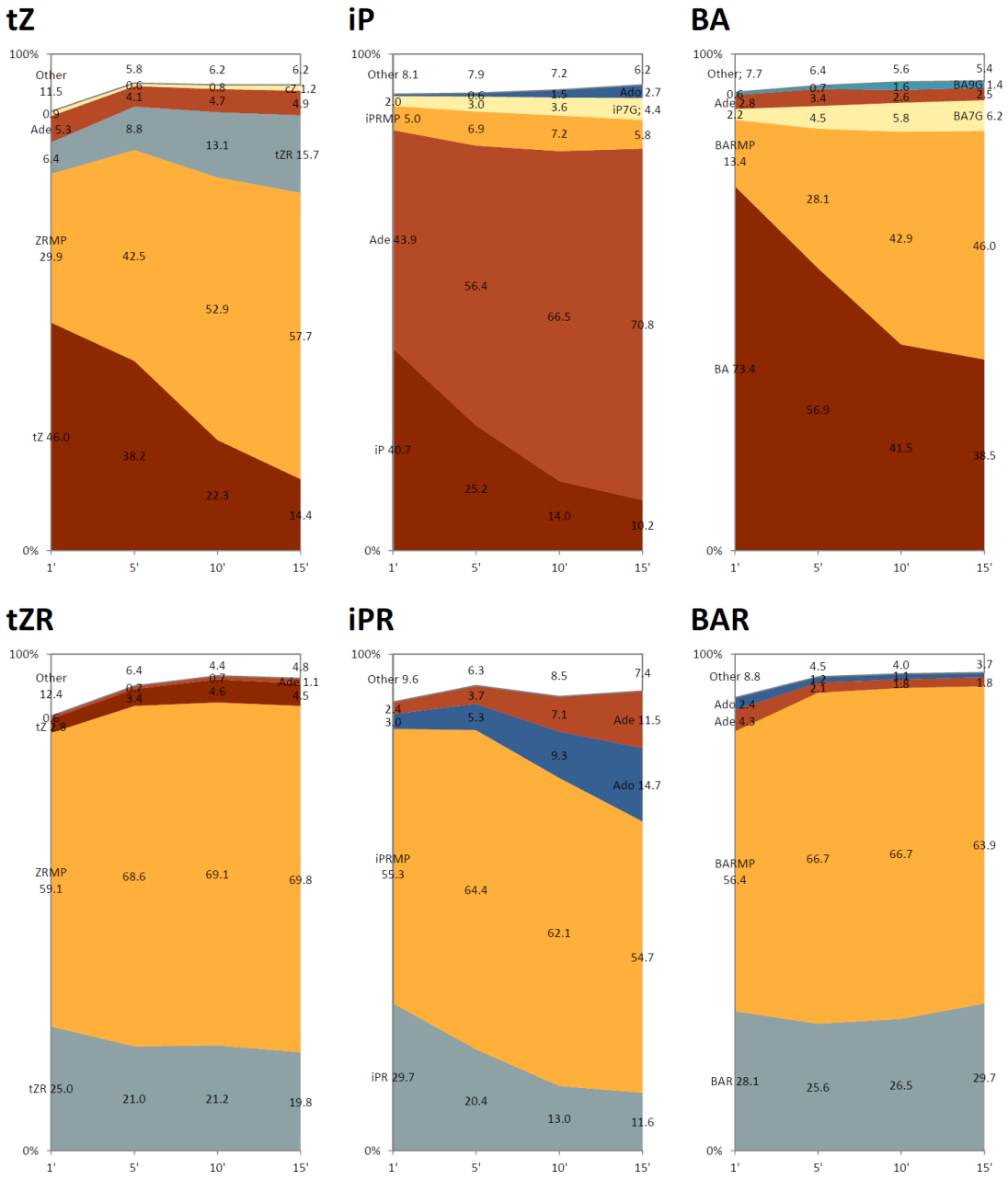

Figure 19 Quantification of detected metabolites. Areas of metabolite peaks were integrated and expressed as percentual part of the total area of the corresponding plot to highlight the relative proportions and time-dependent dynamic changes in detected metabolite levels. 100 % = total integrated area per single run. Ade (adenine), Ado (adenosine), tZ (*trans*-zeatin), cZ (*cis*-zeatin), iP (isopentenyladenine), BA (benzyladenine), tZR (*trans*-zeatin riboside), iPR (isopentenyladenosine), BAR (benzyladenosine), ZRMP (*trans*-zeatin riboside monophosphate), iPRMP (isopentenyladenosine monophosphate), BARMP (benzyladenosine monophosphate), iP7G (isopentenyladenine 7-glucoside), BA7G (benzyladenine 7-glucoside), BA9G (benzyladenine 9-glucoside), Other (non-identified peaks or metabolites under 1 % of relative abundance).

6.5 Cytokinin oxidase/dehydrogenase activity assay

To check whether the accumulation of CK-bases and their subsequent degradation to adenine is accompanied by an increase in cytokinin oxidase/dehydrogenase (CKX) activity, BY-2 cells were treated with 2 nM, 20 nM, 200 nM and 20 µM concentrations of cold tZ and iP mimicking an actual accumulation experiment. Samples of 1 g (FW) of suspension cells were removed in the course of the experiment at 5 minutes intervals up to 20 minutes. Change of CKX activity was determined in an assay based on conversion of 2 µM [^{3}H]iP to [^{3}H]Ade as described in methods. The enzyme preparation containing 0.7 to 1.2 mg protein × g^{-1} FW (equivalent to 10 mg of tissue FW) allowed the optimum degradation of [^{3}H]iP in the range of 15-55 %.

Both tZ and iPA seem to modify the CKX activity in BY-2 cell culture, although differently (**Table 3**). Except the 5 minute timepoint, tZ at the lowest tested concentration induces an increase of CKX activity by up to 50 % compared to control. On the other hand, higher concentrations of tZ rather diminish CKX activity. The most prominent example is the inhibition by as much as 30 % in the 20 nM and 20 µM tZ addition variants. iP applied to the cells in concentration range from 2 nM to 200 nM did not modify the CKX activity by more than 13 % and in most cases its increase rather than decrease was recorded. However, feeding BY-2 cells with 20 µM iP resulted in a gradual decrease down to 46 % of the remaining CKX activity on the last, 20 minutes sampling.

6.5.1 CKX activity and substrate specificity assay in BY-2

The difference in metabolic inactivation between iP, which was preferentially degraded to Ade, and tZ, which was deactivated by formation of tZRMP, led us to investigate the substrate specificity of crude extract of CKX in BY-2 cells.

For this purpose, the enzyme preparation from the "iP 20 µM" control sample from previous experiment was used. The enzyme preparation equivalent to 20 mg of tissue FW (0.91 mg protein × g^{-1} FW) was used to test the substrate specificity for [^{3}H]tZ, [^{3}H]iP and [^{3}H]cZ. The order of preference for potential substrates was determined as iP > cZ > tZ in BY-2 cells (**Table 2**).

Substrate	CKX activity [nmol Ade × mg⁻1 protein × h⁻1]			Relative to iP
	1st replicate	2nd replicate	ø	(%)
iP	0.539	0.490	0.515	100
tZ	0.079	0.084	0.082	16
cZ	0.361	0.282	0.322	62

Table 2 Substrate specificity of crude extracts of CKX enzymes from tobacco BY-2 cells toward iP, tZ and cZ. The CKX activity was determined using tritiated substrates [^{3}H]iP, [^{3}H]tZ and [^{3}H]cZ, 2 µM each, as substrates.

Treatment	Concentration	Time [mm:ss]	Protein content [mg × g⁻1 FW]	CKX activity [nmol Ade × mg⁻1 protein × h⁻1]			
				1st replicate	2nd replicate	ø	Δ control [%]
tZ	2 nM	Control	0.856	0.520	0.356	0.438	100.0
		0:34	0.930	0.558	0.473	0.516	117.7
		5:17	0.817	0.306	0.424	0.365	83.3
		10:18	0.868	0.570	0.753	0.662	151.0
		15:20	0.762	0.648	0.617	0.633	144.4
		20:18	0.788	0.582	0.735	0.659	150.3
	20 nM	Control	0.500	1.225	0.886	1.056	100.0
		0:34	0.569	1.093	1.091	1.092	103.5
		5:17	0.734	0.795	0.721	0.758	71.8
		10:19	0.838	0.744	0.672	0.708	67.1
		15:19	0.938	0.734	0.740	0.737	69.8
		20:16	0.684	0.919	0.865	0.892	84.5
	200 nM	Control	0.671	0.654	0.677	0.666	100.0
		0:32	0.920	0.548	0.554	0.551	82.8
		5:15	0.853	0.738	0.755	0.747	112.2
		10:13	0.913	0.751	0.596	0.674	101.2
		15:17	0.774	0.619	0.601	0.610	91.7
		20:10	0.797	0.608	0.656	0.632	95.0
	20 µM	Control	1.093	0.486	0.498	0.492	100.0
		0:32	0.903	0.462	0.423	0.443	89.9
		5:22	0.944	0.398	0.430	0.414	84.1
		10:15	1.096	0.390	0.381	0.386	78.4
		15:15	1.000	0.369	0.327	0.348	70.7
		20:09	1.023	0.353	0.388	0.371	75.3
iP	2 nM	Control	0.808	0.593	0.601	0.597	100.0
		0:30	1.027	0.643	0.677	0.660	110.6
		5:13	0.946	0.570	0.549	0.560	93.7
		10:15	1.008	0.644	0.638	0.641	107.4
		15:12	0.937	0.564	0.629	0.597	99.9
		20:15	0.977	0.663	0.664	0.664	111.1
	20 nM	Control	0.887	0.594	0.610	0.602	100.0
		0:32	1.076	0.579	0.641	0.610	101.3
		5:04	1.214	0.566	0.518	0.542	90.0
		10:07	1.130	0.583	0.586	0.585	97.1
		15:12	1.001	0.668	0.661	0.665	110.4
		20:13	0.882	0.587	0.565	0.576	95.7
	200 nM	Control	0.901	0.526	0.512	0.519	100.0
		0:35	0.823	0.583	0.525	0.554	106.7
		5:10	0.998	0.557	0.493	0.525	101.2
		10:10	0.901	0.538	0.638	0.588	113.3
		15:11	0.821	0.543	0.457	0.500	96.3
		20:20	0.882	0.586	0.472	0.529	101.9
	20 µM	Control	0.911	0.637	0.589	0.613	100.0
		0:32	1.115	0.475	0.522	0.499	81.3
		5:07	0.946	0.411	0.338	0.375	61.1
		10:25	1.093	0.357	0.368	0.363	59.1
		15:11	0.875	0.370	0.383	0.377	61.4
		20:09	1.219	0.347	0.313	0.330	53.8

Table 3 CKX activity assay. The enzymatic activity of CKX was checked in BY-2 cells in four timepoints during the accumulation of tZ and iP at various concentrations (2 nM, 20 nM, 200 nM and 20 µM). Control samples were removed before the addition of cold CKs. Samples (1 g FW) were removed in the first, fifth, tenth and fifteenth minute of the accumulation experiment and CKX activity was determined. Mean of two replicates of CKX activity assessment is given in the table and the percentual change compared to the control value (100 %) is represented in shades of blue (less than 100 % activity) and red (more than 100 % activity).

7. Discussion

7.1 Cytokinins accumulate rapidly in BY-2 cells

I have shown throughout this study that BY-2 cultured cells are, in addition to reliable measuring of auxin transport, a suitable model for assessing the short-term kinetics of CK transport at the plant cellular level. All the convenience and reliability of BY-2 cell culture as a plant model comes as a bonus for transport studies: its high growth rate and high homogeneity being among the most prominent advantages. Moreover, its friability allows to express the accumulation rates per cell number indeed.

There have been several attempts to describe the absorption or excretion of CKs using plant suspension-grown cells of several tobacco cultivars like Wisconsin 38 (Laloue et al., 1977; Terrine and Laloue, 1980; Motyka et al., 2003), VBI-0 (Petrášek et al., 2002) or even BY-2 itself (Mlejnek et al., 2003, 2005); and recently also in *Arabidopsis* (Cedzich et al., 2008). However, the majority of experiments characterized the kinetics of CK translocation on hours or even days spreading timescale. I tried to focus more on short term direct uptake measurement of selected bioactive CKs, using tritium labelling as a tracer.

The accumulation assays using BY-2 cells revealed a quick uptake of ^{3}H-labelled t*Z*, iP, BA, and their respective riboside forms: tZR, iPR and BAR. In an attempt to investigate the early accumulation stage, linear uptake kinetics was recorded during the first 10 minutes of the accumulation run of tZR, iPR and BA, after the offset caused probably by initial cell wall contamination. This artifact is eliminated in further assays by subtracting the first collected aliquots as correction. Detected uptake linearity is in accordance with results by Cedzich et al. (2008) who assessed tZ transport kinetics in *Arabidopsis* suspension culture in 4 minutes uptake experiment. However, in my case tZ, iP and BAR show a non-linear absorption tendency. This fact becomes even more obvious during a longer accumulation assay where tZ, iP, iPR and BAR accumulation rates clearly slow down at later stages.

Similar uptake kinetics would support earlier views on CKs transport which was considered to be a simple diffusion. Actually, CK-bases and CK-ribosides under physiological pH are non-polar lipophilic molecules (Horgan and Scott, 1987; Barták et al., 2000) that could potentially cross the phospholipid membranes dependently on concentration gradient. However, recent advances in identification of proteins capable of CK translocation (Gillissen et al., 2000; Cedzich et al., 2008;

Bürkle et al., 2003; Sun et al., 2005; Hirose et al., 2005, 2008) support the hypothesis of an active transport process.

7.2 Kinetics of CK uptake in BY-2 cells

To assess a possible involvement of active transport, a series of experiments testing the competition of radiolabelled CK tracers with 'cold' CKs for a shared transport route was performed. Displacement by increasing concentration of the competitor of the same species revealed a saturation of transport of both bases, tZ and iP, and their ribosides in the presence of surplus of the competitor. Approximate displacement constants (K_d), which in the sense of Michaelis-Menten kinetics correspond to K_M of the tested substrate uptake, were estimated to be 100 nM for tZ and 1 µM or higher for tZR and iPR. 100 nM K_d value for tZ is close to K_M predicted by Cedzich et al. (2008) for high-affinity tZ uptake in *Arabidopsis* cultured cells. Such affinity for a putative transporter would be favorable in the context of 1 nM - 200 nM concntration range of CKs that are usually referred as physiological in literature (Terrine and Laloue, 1980; Komor et al., 1993; Hwang and Sheen, 2001).

The existence of another, low-affinity mode of transport was also reported by Cedzich et al. (2008), and two additional K_m values within the micromolar range (3.9 µM and 98.8 µM) were detected for tZ uptake supplied at concentration 20 µM. The former K_M corresponds to the estimated K_d for tZR and iPR uptake in BY-2 cells. The latter K_M value may be questionned in the light of aforementioned physiological concentrations of CKs in plants. However, the low-affinity transport is comparable with low affinities for CKs that have been described for some enzymes involved in biosynthesis and metabolism of CKs, namely *O*-glycosyl- and *O*-xylosyltransferase in *Phaseolus* (Turner et al., 1987; Dixon et al., 1989), *cis*Z *O*-glucosyltransferase in maize (Veach et al., 2003), CYP735A in *Arabidopsis* (Takei et al., 2004), cytokinin oxidase/dehydrogenase in maize (Bilyeu et al., 2001), as well as enzymes of the purine salvage pathway like adenosine kinase (Moffatt et al., 2000) and adenine phosphoribosyltransferase in *Arabidopsis* (Allen et al., 2002). Moreover, the existence of low-affinity CK transport is supported by identification of both PUPs and ENTs as low-affinity CK transporters.

Consistently with data published on the mechanism of solute transport by AtPUPs (Gillissen et al., 2000; Bürkle et al., 2003), AtENTs and OsENTs (Möhlmann et al., 2001; Wormit et al., 2004; Hirose et al., 2005), and general uptake of CKs in *Arabidopsis* suspension-grown cells (Cedzich et al., 2008), the transport of tZ, iP, tZR and iPR is dependent on proton gradient in BY-2 cells. CCCP, the uncoupler of oxidative phosphorylation, effectively abolishes the uptake and possible

excretion of CKs or their metabolites. This is probably the main argument against a simple diffusion as a driving force of the transmembrane transport of CKs.

7.3 Substrate specificity of CK transport in BY-2

The potential existence of a common transport systems for adenine and CKs, or nucleosides and CKs, has been anticipated by competitive inhibition of adenine or adenosine uptake by a number of CK-bases and CK-ribosides in yeast, transformed with *AtPUP1*, *AtPUP2*, *AtENT3*, *AtENT6* and *OsENT2* (Gillissen et al., 2000; Bürkle et al., 2003; Hirose et al., 2005, 2008), as well as in wild-type *Arabidopsis* suspension cells (Bürkle et al., 2003; Cedzich et al., 2008).

My experiments using wild-type BY-2 cell culture revealed a strong competition for uptake between [^{3}H]tZ or [^{3}H]BA and cold CK-bases, including iP, DHZ, Kin; and also Ade. [^{3}H]iP was an exception as the competition with other cold CK-bases was lower and, interestingly, Ade showed only very weak interference with iP uptake. This means that tested CK-bases, except iP, may share a common way of translocation over the plasma membrane. Because of the fact that iP inhibited more effectively the uptake of both [^{3}H]tZ and [^{3}H]BA than vice-versa, it is conceivable that iP enters BY-2 cells via two distinct routes: one is the shared CK-bases/Ade way and the other one is a unique translocation way for iP.

Regarding CK-ribosides, high surplus of all tested cold CK-riboside competitors significantly lowered the accumulation of all three [^{3}H]CK-ribosides. Yet, Ado was the only substance, which did not perturb the accumulation rate of CK-ribosides at all. Surprisingly, Ado as well as some CK-ribosides were good competitors for [^{3}H]CK-bases (tZ and BA) uptake.

Conversely some CK-bases like iP or BA disturbed [^{3}H]tZR and [^{3}H]BAR absorption in BY-2 cells, although to a smaller extent. These results correspond to the findings published by Cedzich et al. (2008) who also reported the competition of tZR for [^{3}H]tZ uptake in *Arabidopsis* cultured cells; or to the results of Gillissen et al. (2000), Bürkle et al. (2003) and Hirose et al. (2005), who reported on the specificities of single PUP and ENT transporters of *Arabidopsis* and rice origin using heterologous expression in yeast. Altogether, these specificity tests imply a probable existence of at least two transport systems: one which may have overlapping specificity for CK-bases and CK-ribosides, and one which is specific for CK-ribosides.

Interestingly, neither Ade nor Ado perturbed the accumulation rate of CK-ribosides. Instead, both substances were shown to compete for the same transport route with CK-bases in BY-2. This fact suggests the existence of a yet uncharacterized separate transporter specific for either

CK-ribosides or Ado, and a shared transporter for CK-bases, Ade and Ado. Members of the PUP family may account for candidates for such function: 1) So far, only AtPUP1-3 of the large 21 members protein family have been characterized, 2) AtPUP1 has been shown to facilitate the transport of adenine, adenosine, CK-bases and limitedly also CK-ribosides (Gillissen et al., 2000; Bürkle et al., 2003), and 3) the transport of Ade by AtPUP2 is inhibited by both CK-bases (tZ, cZ, iP, BA) and tZR (Bürkle et al., 2003).

7.4 After uptake: A fast metabolism of CKs

CK levels in plant cells depend on several factors, including CK biosynthesis, metabolic interconversions, inactivation, degradation as well as uptake and excretion (Kamínek et al., 1997). External application of biologically active CKs leads to their accumulation inside plant cells which is accompanied by their progressive degradation or metabolic inactivation (Laloue et al., 1977; Terrine and Laloue, 1980; Fusseder and Ziegler, 1988; Mlejnek et al., 2003, 2005; Gaudinová et al., 2005).

HPLC-based metabolic profiling of radiolabelled tZ, iP, BA and their ribosides based on a short-term feeding experiment in BY-2 cells revealed that the intracellular metabolic inactivation of CK is an immediate process which was not reported previously. During 15 minutes of the accumulation experiment with 20 nM CK substrates, more than 80 % of the total tracer radioactivity relocated from the monitored CK into a spectrum of metabolites. In most cases, the metabolic flow was steep right from the beginning of the experiment and usually only one metabolite prevailed. With the exception of iP which was degraded preferably to Ade, an appropriate CK monophosphate was formed at the expense of delivered [^{3}H]CK-bases (tZ, cZ, BA) and [^{3}H]CK-ribosides (tZR, iPR, BAR). Based on this observation, only 3 enzymes are involved in this first answer to excessive CK abundance: cytokinin oxidase/dehydrogenase, adenosine kinase and phosphoribosyl transferase.

In general these results are in accord with already published work. Laloue et al. (1977) detected Ade and various adenylic nucleotides as primary metabolites of iP and iPR fed to tobacco suspension cells. Mlejnek et al. (2003, 2005) observed CK-riboside monophosphates and *N*7-glucosides as main metabolites of BA, BAR and iP in BY-2 cells. Interestingly, no degradation of iP to Ade was reported in this case. This difference may be explained by the high concentration (10-50 µM) of CKs used in the experiment or by the extended experimental timeframe.

Excessive formation of CK-nucleotides is known to deplete the pool of available ATP and consequently to lead to programmed cell death (Mlejnek et al., 2003, 2005). In this regard, the formation of CK-ribotides may be considered as a "first aid" action deployed readily to maintain cell's hormonal homeostasis. In order to prevent a possible collapse of intracellular metabolism, a shift to "harmless" metabolites needs to occur. Despite the limited experimental time course, such trend becomes apparent in metabolic profiles of [^{3}H]iP and [^{3}H]BA, where levels of 7- and 9-*N*-glucosides gradually increase. Similar progress may be seen in the continuously growing Ade/Ado portion in metabolic profiles of [^{3}H]cZ and [^{3}H]iPR.

The striking preference for [^{3}H]iP degradation to adenine, as well as increasing ratios of adenine or adenosine in metabolic profiles of [^{3}H]cZ and [^{3}H]iPR are supported by differential substrate specificity (iP>cZ>tZ) of crude extract of CKX enzymes from tobacco BY-2 cells. Eventhough CKX specificity towards iPR was not determined in BY-2, both iP and its riboside are known as preferred substrates of CKX (Kamínek, 1992).

CKX activity in tobacco cv. Wisconsin 38 suspension-grown cells is enhanced by either exogenous CK application or conditional *IPT* expression after 4 days of treatment (Motyka et al., 2003). Our results show, that low concentrations of tZ or iP may enhance CKX activity by 50 % in the course of 20 min experiment. Contrary, higher concentrations (tZ: 20 nM-20 µM; iP: 20 µM) may inhibit its activity down to 50 % compared to control. Together with the detected affinity of CKX extract towards tZ, this would explain why 20 nM [^{3}H]tZ was not degraded to adenine, and also why higher concentration of iP was primarily metabolized to iPRMP instead of Ade (Mlejnek et al., 2005).

7.5 Physiological implications

CKs are signal molecules that can act at the site of their production or they can transfer the information about a particular physiological status to a distant part of the plant body. Contrary to a directed flow of auxin via PIN or ABC transporters, no such mechanism has been discovered for CKs yet. According to the present state of the art, any crossing of the plasmalemma by CKs seems to be facilitated by a set of purine permeases or nucleoside transporters with broad substrate specificities. Not all the members of ENT or PUP families have been characterized yet in terms of CK transport ability, some of them have not been even cloned yet, so the possibility of existence of a CK-specific transporter among PUPs and ENTs is not excluded, as well as the existence of other, yet unknown transport systems.

The question of the need of plant cells to take up CKs has not been answered fully up to now. From the data published in this thesis and from results by others, it is possible to conclude that the purpose of CK absorption consists in the necessity of metabolic inactivation of the biologically active signalling molecules. Eventhough CKX activity occurs outside of the cell, other enzymes indispensable for CK metabolism, including biosynthesis, are found in various inner cell compartments. In this regard, the need for internal transmembrane transport of CKs arises as well but, to the best of my knowledge, was not adressed yet. Together with the necessity of the newly synthesized CKs to leave cytoplasmic space, the need for a transport system is obvious.

The link between the transport of CKs and their fast inactivation by target cells may constitute a basic principle of a dynamic system where a signalling molecule is synthesized, delivered to target cells and promptly deactivated after signal reception. This sequence may be then continuously reproduced until the reason for signalization event passes away. Most CK concentrations used throughout this thesis are of reported physiological range. This fact validates the results for real *in planta* scenario. The undifferenciated character of the cell suspension then encourages the application of a similarly functioning model, if not to plant cells in general, then at least to meristematic tissues and their response to long distance CK signalling.

So far, CK signalling cascade model assumed that CK receptors reside on the plasma membrane. However, latest work of Wulfetange et al. (2011) brings evidence for intracellular localization of CK receptors, proposing the perception of CK signal to occur at the endoplasmic reticulum. Uptake of CKs by plant cells would be then appointed a new significance.

8. Conclusions

The mechanism of translocation of cytokinins across plasma membrane and their intracellular metabolic fate was investigated using tobacco BY-2 suspension-grown cells. In addition to a reliable auxin transport measuring, BY-2 cell culture has proven to be a capable model system for CK transport experiments using tritiated CKs at their physiological concentrations.

Based on observations presented in this thesis, following conclusions can be made:

- Both CK-bases and CK-ribosides are readily taken up by BY-2 tobacco cells. Using competition approach with displacement by the same species of competitor, apparent K_d values for tZ of 100 nM and for tZR and iPR of approximately 1 µM could be estimated. The affinities of putative CK transport systems in BY-2 are compatible with reported concentration range of CKs in plants.
- The substrate specificity assay of CK uptake in BY-2 cells confirmed the existence of a shared uptake route for CK-bases, and also a strong competition for a shared route between CK-ribosides. Surprisingly, some CK-ribosides proved to be good competitors for CK-bases uptake. Adenine and adenosine disturbed the accumulation of CK-bases, but not of CK-ribosides. These observations suggest the existence of a yet unknown carrier(s) with overlapping specificity for CK-bases, Ade and Ado, as well as a specific carrier for CK-ribosides.
- The uptake of both CK-bases and CK-ribosides by BY-2 cells was sensitive to CCCP, indicating that it is mediated by secondary active transport systems dependent on plasma mebrane proton gradient.
- HPLC analysis of the metabolites of accumulated CKs pointed at fast degradation of CKs or their metabolic conversion into physiologically inactive forms. Except iP, which was preferably degraded to Ade, an appropriate CK-riboside monophosphate is formed as a first response to increased CK uptake in BY-2 cells. 3 enzymes are likely to be involved in this homeostasis-keeping process: cytokinin oxidase/dehydrogenase, adenosine kinase and phosphoribosyl transferase.
- By analysis of crude extracts of CKX enzymes from BY-2, their highest affinity towards iP was confirmed and their activity was found to be dependent on concentration of externally added bioactive CK.

Závěry

Tato práce se věnuje otázce mechanismu transportu rostlinných hormonů cytokininů a jejich následnému metabolismu v buněčné suspenzi tabáku BY-2. BY-2 se, vedle již osvědčeného použití pro měření transportu auxinu, ukázala být vhodným modelovým systémem i pro experimenty s cytokininy značenými triciem, a to s použitím jejich fyziologických koncentrací.

Na základě výsledků, které jsou v této práci představeny, lze vyvodit následující závěry:

- Báze i ribozidy cytokininů jsou buňkami tabákové suspenzní kultury BY-2 rychle absorbovány. Pomocí kompetičních vytěsňovacích experimentů za použití kompetitoru stejného druhu se zvyšující se koncentrací mohly být odhadnuty hodnoty vytěsňovacích konstant K_d pro tZ v oblasti 100 nM a pro tZR a iPR přibližně 1 µM. Tyto koncentrace jsou shodné s běžně pozorovanými fyziologickými koncentracemi cytokininů v rostlinách.
- Pokusy zkoumající substrátovou specificitu předpokládaných cytokininových přenašečů v suspenzi BY-2 potvrdily kompetici mezi jednotlivými druhy CK bází o stejnou transportní cestu, stejně jako v případě jednotlivých CK ribozidů. Zajímavé zjištění přinesl fakt, že některé ribozidy cytokininů dobře kompetovaly s příjmem cytokininových bází. Dále se ukázalo, že adenin i adenosin interferují s příjmem CK bází a nikoli ribozidů. Dohromady tato zjištění podporují existenci dosud neznámých přenašečů s překrývající se specificitou pro báze cytokininů, adenin a adenosin, případně přenašeče specifického pro cytokininové ribozidy.
- Příjem bází i ribozidů cytokininů buňkami BY-2 byl senzitivní k CCCP, což naznačuje, že jejich přenos je umožněn způsobem sekundárního aktivního transportu, závislého na gradientu protonů na buněčné membráně.
- HPLC analýza cytokininů přijatých buňkami BY-2 poukázala na jejich rychlou degradaci anebo metabolickou přeměnu na biologicky neaktivní formu. S výjimkou iP, který byl přednostně degradován na adenin, podléhaly všechny ostatní testované CK metabolické přeměně na odpovídající nukleotid monofosfát. Na této první homeostatické reakci na zvýšený obsah CK se tedy nejspíše podílely 3 enzymy: cytokinin oxidáza/dehydrogenáza, adenozin kináza a fosforibozyl transferáza.
- Analýza celkové enzymatické aktivity CKX izolované z buněk BY-2 potvrdila vysokou afinitu k iP a závislost míry aktivity na koncentraci externě dodaného biologicky aktivního cytokininu.

9. References

Akiyoshi, D.E. et al. (1984). T-DNA of Agrobacterium tumefaciens encodes an enzyme of cytokinin biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 5994-8.

Allen, M. et al. (2002). Adenine phosphoribosyltransferase isoforms of Arabidopsis and their potential contributions to adenine and cytokinin metabolism. *Physiologia plantarum* **115**: 56-68.

Aloni, R. et al. (2005). Root-synthesized cytokinin in Arabidopsis is distributed in the shoot by the transpiration stream. *Journal of experimental botany* **56**: 1535-44.

Argueso, C.T. et al. (2009). Environmental perception avenues: the interaction of cytokinin and environmental response pathways. *Plant, cell & environment* **32**: 1147-60.

Astot, C. et al. (2000). An alternative cytokinin biosynthesis pathway. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 14778-83.

Bano, A. et al. (1994). Changes in the contents of free and conjugated abscisic acid, phaseic acid and cytokinins in xylem sap of drought stressed sunflower plants. *Phytochemistry* **37**: 345-347.

Barciszewski, J. et al. (2007). Kinetin--a multiactive molecule. *International journal of biological macromolecules* **40**: 182-92.

Barnes, M. (1980). Biosynthesis of cytokinins by potato cell cultures. *Phytochemistry* **19**: 409-412.

Barry, G.F. et al. (1984). Identification of a cloned cytokinin biosynthetic gene. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 4776-80.

Barták, P. et al. (2000). Determination of dissociation constants of cytokinins by capillary zone electrophoresis. *Journal of chromatography. A* **878**: 249-59.

Bassil, N.V. et al. (1993). Partial Purification of a cis-trans-Isomerase of Zeatin from Immature Seed of Phaseolus vulgaris L. *Plant physiology* **102**: 867-872.

Beaty, J.S. et al. (1986). Tzs, a nopaline Ti plasmid gene from Agrobacterium tumefaciens associated with trans-zeatin biosynthesis. *Molecular and General Genetics MGG* **203**: 274-280.

Beveridge, C.A. et al. (1997). The shoot controls zeatin riboside export from pea roots. Evidence from the branching mutant rms4. *The Plant Journal* **11**: 339-345.

Bilyeu, K.D. et al. (2001). Molecular and biochemical characterization of a cytokinin oxidase from maize. *Plant physiology* **125**: 378-86.

Bouadloun, F. et al. (1986). Influence of modification next to the anticodon in tRNA on codon context sensitivity of translational suppression and accuracy. *Journal of bacteriology* **166**: 1022-7.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Brenner, W.G. et al. (2005). Immediate-early and delayed cytokinin response genes of Arabidopsis thaliana identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. *The Plant journal* **44**: 314-33.

Brzobohaty, B. et al. (1993). Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. *Science* **262**: 1051-1054.

Bürkle, L. et al. (2003). Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of Arabidopsis. *The Plant journal* **34**: 13-26.

Cedzich, A. et al. (2008). Characterization of cytokinin and adenine transport in Arabidopsis cell cultures. *Plant physiology* **148**: 1857-67.

Chatfield, J.M. and Armstrong, D.J. (1986). Regulation of Cytokinin Oxidase Activity in Callus Tissues of Phaseolus vulgaris L. cv Great Northern. *Plant physiology* **80**: 493-9.

Chen, C.M. and Petschow, B. (1978). Metabolism of cytokinin: ribosylation of cytokinin bases by adenosine phosphorylase from wheat germ. *Plant physiology* **62**: 871-4.

Corbesier, L. et al. (2003). Cytokinin levels in leaves, leaf exudate and shoot apical meristem of Arabidopsis thaliana during floral transition. *Journal of experimental botany* **54**: 2511-7.

Dixon, S.C. et al. (1989). Zeatin Glycosylation Enzymes in Phaseolus: Isolation of O-Glucosyltransferase from P. lunatus and Comparison to O-Xylosyltransferase from P. vulgaris. *Plant physiology* **90**: 1316-21.

Dobrev, P. et al. (2002). Transient accumulation of cis- and trans-zeatin type cytokinins and its relation to cytokinin oxidase activity during cell cycle of synchronized tobacco BY-2 cells. *Plant Physiology and Biochemistry* **40**: 333-337.

Dobrev, P.I. and Kamínek, M. (2002). Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction . *Journal of chromatography. A* **950**: 21-9.

Dorée, M. and Guern, J. (1973). Short-time metabolism of some exogenous cytokinins in Acer pseudoplatanus cells. *Biochimica et Biophysica Acta* **304**: 611-622.

Faiss, M. et al. (1997). Conditional transgenic expression of the ipt gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. *The Plant Journal* **12**: 401-415.

Foo, E. et al. (2007). Feedback regulation of xylem cytokinin content is conserved in pea and Arabidopsis. *Plant physiology* **143**: 1418-28.

Frébort, I. et al. (2002). Cytokinin oxidase/cytokinin dehydrogenase assay: optimized procedures and applications. *Analytical biochemistry* **306**: 1-7.

Fusseder, A. and Ziegler, P. (1988). Metabolism and compartmentation of dihydrozeatin exogenously supplied to photoautotrophic suspension cultures of Chenopodium rubrum. *Planta* **173**: 104-109.

Gajdošová, S. et al. (2011). Distribution, biological activities, metabolism, and the conceivable function of cis-zeatin-type cytokinins in plants. *Journal of experimental botany* **62**: 2827-40.

Galuszka, P. et al. (2000). Degradation of cytokinins by cytokinin oxidases in plants. *Plant Growth Regulation* **32**: 315-327.

Gaudinová, A. et al. (2005). The Involvement of Cytokinin Oxidase/Dehydrogenase and Zeatin Reductase in Regulation of Cytokinin Levels in Pea (Pisum sativum L.) Leaves. *Journal of Plant Growth Regulation* **24**: 188-200.

Gillissen, B. et al. (2000). A new family of high-affinity transporters for adenine, cytosine, and purine derivatives in Arabidopsis. *The Plant cell* **12**: 291-300.

Grayburn, W.S. et al. (1982). Bud Induction with Cytokinin : A LOCAL RESPONSE TO LOCAL APPLICATION. *Plant physiology* **69**: 682-6.

Hanano, S. et al. (2006). Multiple phytohormones influence distinct parameters of the plant circadian clock. *Genes to cells* **11**: 1381-92.

Hanuš, J. et al. (2000). N6-alkyladenosines and adenines labelled with tritium. *Journal of Labelled Compounds and Radiopharmaceuticals* **43**: 523-531.

Hare, P. et al. (1997). The involvement of cytokinins in plant responses to environmental stress. *Plant Growth Regulation* **23**: 79–103.

Hirose, N. et al. (2005). Functional characterization and expression analysis of a gene, OsENT2, encoding an equilibrative nucleoside transporter in rice suggest a function in cytokinin transport. *Plant physiology* **138**: 196-206.

Hirose, N. et al. (2008). Regulation of cytokinin biosynthesis, compartmentalization and translocation. *Journal of experimental botany* **59**: 75-83.

Horgan, R. and Scott, I.M. (1987). Cytokinins. In The Principles and Practice of Plant Hormone Analysis, Vol. 2, L. Rivier and A. Crozier, eds (Academic Press: London), pp. 303-365.

Horgan, R. et al. (1975). A new cytokinin from Populus x robusta. *Phytochemistry* **14**: 1005-1008.

Hoth, S. et al. (2003). Monitoring genome-wide changes in gene expression in response to endogenous cytokinin reveals targets in Arabidopsis thaliana☆. *FEBS Letters* **554**: 373-380.

Hou, B. et al. (2004). N-glucosylation of cytokinins by glycosyltransferases of Arabidopsis thaliana. *The Journal of biological chemistry* **279**: 47822-32.

Hoyerová, K. et al. (2006). Efficiency of different methods of extraction and purification of cytokinins. *Phytochemistry* **67**: 1151-9.

Hutchison, C.E. et al. (2006). The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *The Plant cell* **18**: 3073-87.

Hwang, I. and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. *Nature* **413**: 383-9.

Hyde, R.J. et al. (2001). The ENT family of eukaryote nucleoside and nucleobase transporters: recent advances in the investigation of structure/function relationships and the identification of novel isoforms. *Molecular Membrane Biology* **18**: 53-63.

Inoue, T. et al. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. *Nature* **409**: 1060-3.

Kakimoto, T. (2001). Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. *Plant and Cell Physiology* **42**: 677-85.

Kamada-Nobusada, T. and Sakakibara, H. (2009). Molecular basis for cytokinin biosynthesis. *Phytochemistry* **70**: 444-9.

Kamínek, M. (1974). Evolution of tRNA and origin of the two positional isomers of zeatin. *Journal of theoretical biology* **48**: 489-92.

Kamínek, M. (1992). Progress in cytokinin research. *Trends in Biotechnology* **10**: 159-164.

Kamínek, M. et al. (1997). Regulation of cytokinin content in plant cells. *Physiologia Plantarum* **101**: 689-700.

Kamínek, M. et al. (1979). Effect of stereospecific hydroxylation of N6-(Δ2-Isopentenyl)adenosine on cytokinin activity. *Planta* **145**: 239-243.

Kasahara, H. et al. (2004). Distinct isoprenoid origins of cis- and trans-zeatin biosyntheses in Arabidopsis. *The Journal of biological chemistry* **279**: 14049-54.

Komor, E. et al. (1993). Loading and translocation of various cytokinins in phloem and xylem of the seedlings of Ricinus communis L. *Planta* **191**: 252-255-255.

Kudoyarova, G.R. et al. (2007). Effect of partial rootzone drying on the concentration of zeatin-type cytokinins in tomato (Solanum lycopersicum L.) xylem sap and leaves. *Journal of experimental botany* **58**: 161-8.

Kurakawa, T. et al. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* **445**: 652-5.

Kuroha, T. et al. (2002). A trans-zeatin riboside in root xylem sap negatively regulates adventitious root formation on cucumber hypocotyls. *Journal of Experimental Botany* **53**: 2193-2200.

Kuroha, T. et al. (2009). Functional analyses of LONELY GUY cytokinin-activating enzymes reveal the importance of the direct activation pathway in Arabidopsis. *The Plant cell* **21**: 3152-69.

Lagerstedt, H.B. and Langston, R.G. (1967). Translocation of Radioactive Kinetin. *Plant physiology* **42**: 611-622.

Laloue, M. et al. (1977). Cytokinins: Metabolism and Biological Activity of N-(Delta-Isopentenyl)adenosine and N-(Delta-Isopentenyl)adenine in Tobacco Cells and Callus. *Plant physiology* **59**: 478-83.

Laureys, F. et al. (1998). Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. *FEBS letters* **426**: 29-32.

Laureys, F. et al. (1999). A low content in zeatin type cytokinins is not restrictive for the occurrence of G1/S transition in tobacco BY-2 cells. *FEBS letters* **460**: 123-8.

Letham, D. et al. (1975). Regulators of cell division in plant tissues XXIII. The identity of an unusual metabolite of 6-benzylaminopurine. *Biochimica et Biophysica Acta (BBA) - General Subjects* **399**: 61-70.

Letham, D.S. (1963). Zeatin, a factor inducing cell division isolated from Zea mays. *Life sciences* **8**: 569-573.

Li, G. et al. (2003). Equilibrative nucleoside transporters of Arabidopsis thaliana. cDNA cloning, expression pattern, and analysis of transport activities. *The Journal of biological chemistry* **278**: 35732-42.

Li, J. and Wang, D. (2000). Cloning and in vitro expression of the cDNA encoding a putative nucleoside transporter from Arabidopsis thaliana. *Plant Science* **157**: 23-32.

Martin, R.C. et al. (2001). A maize cytokinin gene encoding an O-glucosyltransferase specific to cis-zeatin. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 5922-6.

Martin, R.C. et al. (1989). An Enzyme Mediating the Conversion of Zeatin to Dihydrozeatin in Phaseolus Embryos. *Plant Physiology* **90**: 1630-1635.

Martin, R.C., Mok, M.C., and Mok, D.W. (1999a). A gene encoding the cytokinin enzyme zeatin O-xylosyltransferase of Phaseolus vulgaris. *Plant physiology* **120**: 553-8.

Martin, R.C., Mok, M.C., and Mok, D.W. (1999b). Isolation of a cytokinin gene, ZOG1, encoding zeatin O-glucosyltransferase from Phaseolus lunatus. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 284-9.

Miller, C.O. et al. (1956). Isolation, structure and synthesis of kinetin, a substance promoting cell division. *Journal of the American Chemical Society* **78**: 1375–1380.

Miller, C.O. et al. (1955). Structure and synthesis of kinetin. *Journal of the American Chemical Society* **77**: 2662-2663.

Miyawaki, K. et al. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis : tissue specificity and regulation by auxin, cytokinin, and nitrate. *The Plant Journal* **37**: 128-138.

Miyawaki, K. et al. (2006). Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 16598-603.

Mlejnek, P. and Procházka, S. (2002). Activation of caspase-like proteases and induction of apoptosis by isopentenyladenosine in tobacco BY-2 cells. *Planta* **215**: 158-66.

Mlejnek, P. et al. (2003). Intracellular phosphorylation of benzyladenosine is related to apoptosis induction in tobacco BY-2 cells. *Plant, Cell and Environment* **26**: 1723-1735.

Mlejnek, P. et al. (2005). Intracellular conversion of cytokinin bases into corresponding mononucleotides is related to cell death induction in tobacco BY-2 cells. *Plant Science* **168**: 389-395.

Moffatt, B. a et al. (2000). Adenosine kinase of Arabidopsis. Kinetic properties and gene expression. *Plant physiology* **124**: 1775-85.

Mok, M.C. et al. (2005). Topolins and hydroxylated thidiazuron derivatives are substrates of cytokinin O-glucosyltransferase with position specificity related to receptor recognition. *Plant physiology* **137**: 1057-66.

Motyka, V. and Kamínek, M. (1994). Cytokinin oxidase from auxin- and cytokinin-dependent callus cultures of tobacco (Nicotiana tabacum L.). *Journal of Plant Growth Regulation* **13**: 1-9.

Motyka, V. et al. (2003). Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. *Physiologia Plantarum* **117**: 11-21.

Mähönen, A.P., Bishopp, A., et al. (2006). Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. *Science* **311**: 94-8.

Mähönen, A.P. et al. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. *Genes & Development* **14**: 2938-2943.

Mähönen, A.P., Higuchi, M., et al. (2006). Cytokinins regulate a bidirectional phosphorelay network in Arabidopsis. *Current biology* **16**: 1116-22.

Möhlmann, T. et al. (2001). Characterisation of a concentrative type of adenosine transporter from Arabidopsis thaliana (ENT1,At). *FEBS letters* **509**: 370-4.

Nagata, T. et al. (1992). Tobacco BY-2 Cell Line as the “HeLa” Cell in the Cell Biology of Higher Plants. *International Review of Cytology* **132**: 1-30.

Nandi, S. et al. (1989). 6-benzylaminopurine and its glycosides as naturally occurring cytokinins. *Plant Science* **61**: 189-196.

Nishinari, N. and Syōno, K. (1980). Changes in endogenous cytokinin levels in partially synchronized cultured tobacco cells. *Plant physiology* **65**: 437-41.

Van Overbeek, J. et al. (1941). Factors in coconut milk essential for growth and development of very young datura embryos. *Science* **94**: 350-1.

Pačes, V. and Kamínek, M. (1976). Effect of ribosylzeatin isomers on the enzymatic degradation of N6-({Delta}2-isopentenyl) adenosine. *Nucleic Acids Research* **3**: 2309-2314.

Pačes, V. et al. (1971). Conversion of N6-(2-Isopentenyl)adenosine to Adenosine by Enzyme Activity in Tobacco Tissue. *Plant physiology* **48**: 775-778.

Perilli, S. et al. (2010). The molecular basis of cytokinin function. *Current opinion in plant biology* **13**: 21-6.

Persson, B.C. et al. (1994). Synthesis and function of isopentenyl adenosine derivatives in tRNA. *Biochimie* **76**: 1152-60.

Petrášek, J. and Zažímalová, E. (2006). The BY-2 Cell Line as a Tool to Study Auxin Transport. In Tobacco BY-2 Cells: From Cellular Dynamics to Omics, T. Nagata et al., eds, pp. 107-117.

Petrášek, J. et al. (2002). Excretion of cytokinins into the cultivation medium by suspension-cultured tobacco cells. *Plant Cell Reports* **21**: 97-104.

Petrášek, J. et al. (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**: 914-8.

Petrášek, J. et al. (2003). Do phytotropins inhibit auxin efflux by impairing vesicle traffic? *Plant physiology* **131**: 254-63.

Rashotte, A.M. et al. (2003). Expression profiling of cytokinin action in Arabidopsis. *Plant Physiology* **132**: 1998.

Rashotte, A.M. et al. (2006). A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 11081-5.

Redig, P. et al. (1996). Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. *FEBS letters* **391**: 175-80.

Riefler, M. et al. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *The Plant cell* **18**: 40-54.

Rovati, G.E. (1998). Ligand-binding studies: old beliefs and new strategies. *Trends in pharmacological sciences* **19**: 365-9.

Sakakibara, H. (2006). Cytokinins: activity, biosynthesis, and translocation. *Annual review of plant biology* **57**: 431-49.

Sakakibara, H. et al. (2005). Agrobacterium tumefaciens increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 9972-7.

Sakamoto, T. et al. (2006). Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. *Plant physiology* **142**: 54-62.

Samuelson, M.E. et al. (1992). Nitrate-regulated growth and cytokinin responses in seminal roots of barley. *Plant physiology* **98**: 309-15.

Schmülling, T. et al. (2003). Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. *Journal of plant research* **116**: 241-52.

Shantz, E.M. and Steward, F.C. (1955). The identification of compound A from coconut milk as 1, 3-diphenylurea. *Journal of the American Chemical Society* **77**: 6351–6353.

Skoog, F. (1994). A personal history of cytokinin and plant hormone research. In Cytokinins : chemistry, activity, and function, D.W. Mok and M.C. Mok, eds (CRC Press: Boca Raton, Fla. (USA)), pp. 9-14.

Skoog, F. and Armstrong, D.J. (1970). Cytokinins. *Annual Review of Plant Physiology* **21**: 359-384.

Strnad, M. (1997). The aromatic cytokinins. *Physiologia Plantarum* **101**: 674-688.

Strnad, M. et al. (1997). Meta-topolin, a highly active aromatic cytokinin from poplar leaves (Populus × canadensis Moench., cv. Robusta). *Phytochemistry* **45**: 213-218.

Sun, J. et al. (2005). Arabidopsis SOI33/AtENT8 Gene Encodes a Putative Equilibrative Nucleoside Transporter That Is Involved in Cytokinin Transport In Planta. *Journal of Integrative Plant Biology* **47**: 588-603.

Suzuki, T. (2001). The Arabidopsis Sensor His-kinase, AHK4, Can Respond to Cytokinins. *Plant and Cell Physiology* **42**: 107-113.

Takei, K. (2001). Nitrogen-Dependent Accumulation of Cytokinins in Root and the Translocation to Leaf: Implication of Cytokinin Species that Induces Gene Expression of Maize Response Regulator. *Plant and Cell Physiology* **42**: 85-93.

Takei, K. et al. (2001). Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. *The Journal of biological chemistry* **276**: 26405-10.

Takei, K., Ueda, N., et al. (2004). AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. *Plant and Cell Physiology* **45**: 1053-62.

Takei, K., Yamaya, T., et al. (2004). Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-Zeatin. *The Journal of biological chemistry* **279**: 41866-72.

Tanaka, M. et al. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. *The Plant journal* **45**: 1028-36.

Tarkowska, D. et al. (2003). Identification of new aromatic cytokinins in Arabidopsis thaliana and Populus x canadensis leaves by LC-(+)ESI-MS and capillary liquid chromatography/frit-fast atom bombardment mass spectrometry. *Physiologia Plantarum* **117**: 579-590.

Taya, Y. et al. (1978). 5'-AMP is a direct precursor of cytokinin in Dictyostelium discoideum. *Nature* **271**: 545-547.

Terrine, C. and Laloue, M. (1980). Kinetics of N-(Delta-Isopentenyl)Adenosine Degradation in Tobacco Cells: Evidence of a Regulatory Mechanism Under the Control of Cytokinins. *Plant physiology* **65**: 1090-5.

Tirichine, L. et al. (2007). A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. *Science* **315**: 104-7.

To, J.P.C. and Kieber, J.J. (2008). Cytokinin signaling: two-components and more. *Trends in plant science* **13**: 85-92.

To, J.P.C. et al. (2007). Cytokinin regulates type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay. *The Plant cell* **19**: 3901-14.

Turner, J.E. et al. (1987). Isolation and partial purification of an enzyme catalyzing the formation of O-xylosylzeatin in Phaseolus vulgaris embryos. *Proceedings of the National Academy of Sciences of the United States of America* **84**: 3714-7.

Ueguchi, C. (2001). The AHK4 Gene Involved in the Cytokinin-Signaling Pathway as a Direct Receptor Molecule in Arabidopsis thaliana. *Plant and Cell Physiology* **42**: 751-755.

Veach, Y.K. et al. (2003). O-glucosylation of cis-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. *Plant physiology* **131**: 1374-80.

Werner, T. and Schmülling, T. (2009). Cytokinin action in plant development. *Current opinion in plant biology* **12**: 527-38.

Werner, T. et al. (2006). New insights into the biology of cytokinin degradation. *Plant biology* **8**: 371-81.

Werner, T. et al. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *The Plant cell* **15**: 2532-50.

Werner, T. et al. (2001). Regulation of plant growth by cytokinin. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 10487-92.

Wormit, A. et al. (2004). Characterization of three novel members of the Arabidopsis thaliana equilibrative nucleoside transporter (ENT) family. *The Biochemical journal* **383**: 19-26.

Wulfetange, K. et al. (2011). The Cytokinin Receptors of Arabidopsis thaliana are Locating Mainly to the Endoplasmic Reticulum. *Plant physiology*.

Yamada, H. (2001). The Arabidopsis AHK4 Histidine Kinase is a Cytokinin-Binding Receptor that Transduces Cytokinin Signals Across the Membrane. *Plant and Cell Physiology* **42**: 1017-1023.

Yong, J.W. et al. (2000). Effects of elevated [CO(2)] and nitrogen nutrition on cytokinins in the xylem sap and leaves of cotton. *Plant physiology* **124**: 767-80.

10. List of publications

Papers in impacted journals

Gajdošová, S., Spíchal, L., Kamínek, M., Hoyerová, K., Novák, O., Dobrev, P. I., Galuszka, P., **Klíma, P.**, Gaudinová, A., Žižková, E., Hanuš, J., Dančák, M., Trávníček, B., Pešek, B., Krupička, M., Vaňková, R., Strnad, M., Motyka, V. Distribution, biological activities, metabolism, and the conceivable function of *cis*-zeatin-type cytokinins in plants (2011) *Journal of Experimental Botany*, 62 (8), pp. 2827-2840.

Pernisová, M., **Klíma, P.**, Horák, J., Válková, M., Malbeck, J., Souček, P., Reichman, P., Hoyerová, K., Dubová, J., Friml, J., Zažímalová, E., Hejátko, J. Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux (2009) *Proceedings of the National Academy of Sciences of the United States of America*, 106 (9), pp. 3609-3614. Cited 25 times.

Souček, P., **Klíma, P.**, Reková, A., Brzobohatý, B. Involvement of hormones and *KNOXI* genes in early *Arabidopsis* seedling development (2007) *Journal of Experimental Botany*, 58 (13), pp. 3797-3810. Cited 4 times.

Abstracts from international conferences published in special numbers of impact factor journals (first author posters)

Klíma P., Marková L., Zažímalová E. The mechanism of accumulation of cytokinins in suspension-cultured tobacco cells. (2005) *Biologia Plantarum*, 49 (Suppl.): S11.

Other first author posters

Klíma, P., Dobrev, P. I., Hoyerová, K., Kamínek, M., Zažímalová, E. After uptake – A fast metabolism of cytokinins in BY-2 tobacco cells. (2010) IPGSA, Tarragona, Spain.

Klíma, P., Hošek, P., Hoyerová, K., Jiřina, M., Zažímalová, E. Transport of cytokinins in BY-2 suspension-grown tobacco cells – towards a mathematical model. (2009) ACPD, Prague, Czech Republic.

Distribution, biological activities, metabolism, and the conceivable function of *cis*-zeatin-type cytokinins in plants

Gajdošová, S., Spíchal, L., Kamínek, M., Hoyerová, K., Novák, O., Dobrev, P. I., Galuszka, P., **Klíma, P.**, Gaudinová, A., Žižková, E., Hanuš, J., Dančák, M., Trávníček, B., Pešek, B., Krupička, M., Vaňková, R., Strnad, M., Motyka, V.

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This paper is dealing with the still unclear role of *cis*-zeatin in plants. Historically, cZ was reported to be weakly active as CK, yet cZ-type CKs occur in high abundance in some plant species and their levels and proportion to the *trans* isomer may fluctuate during plant ontogenesis. We showed that cZ may trigger signalling events and that it is active in several biotests. Together with resistance against degradation by AtCKX2, which is expressed during senescence, cZ-type CKs seems to be prevalent in developmental stages connected with limited growth and their function may thus consist of maintaining a minimal level of CK response.

My task in this collaborative work was to test the transport characteristics of cZ and tZ in BY-2 tobacco cells. I also collected samples for short-term metabolism assay in BY-2 cells and participated on their subsequent processing. I am commenting the results in Chapters 5.1.3 and 5.4.1.

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RESEARCH PAPER

Distribution, biological activities, metabolism, and the conceivable function of *cis*-zeatin-type cytokinins in plants

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Abstract

Cytokinins (CKs) are plant hormones affecting numerous developmental processes. Zeatin and its derivatives are the most important group of isoprenoid CKs. Zeatin occurs as two isomers: while *trans*-zeatin (*trans*Z) was found to be a bioactive substance, *cis*-zeatin (*cis*Z) was reported to have a weak biological impact. Even though *cis*Z derivatives are abundant in various plant materials their biological role is still unknown. The comprehensive screen of land plants presented here suggests that *cis*Z-type CKs occur ubiquitously in the plant kingdom but their abundance might correlate with a strategy of life rather than with evolutionary complexity. Changing levels of *trans*Z and *cis*Z during *Arabidopsis* ontogenesis show that levels of the two zeatin isomers can differ significantly during the life span of the plant, with *cis*Z-type CKs prevalent in the developmental stages associated with limited growth. A survey of the bioassays employed illustrates mild activity of *cis*Z and its derivatives. No *cis*↔*trans* isomerization, which would account for the effects of *cis*Z, was observed in tobacco cells and oat leaves. Differences in uptake between the two isomers resulting in distinct bioactivity have not been detected. In contrast, *cis*Z and *trans*Z have a different metabolic fate in oat and tobacco. Analysis of a CK-degrading enzyme, cytokinin oxidase/dehydrogenase (CKX), reveals that *Arabidopsis* possesses two isoforms, AtCKX1 expressed in stages of active growth, and AtCKX7, both of which have the highest affinity for the *cis*Z isomer. Based on the present results, the conceivable function of *cis*Z-type CKs as delicate regulators of CK responses in plants under growth-limiting conditions is hypothesized.

Key words: *Arabidopsis thaliana*, *cis*-zeatin, cytokinin, cytokinin oxidase/dehydrogenase, growth-limiting conditions, oat, tobacco BY-2 cells, *trans*-zeatin.

Introduction

Cytokinins (CKs) represent a large group of plant hormones which affect various vital processes throughout plant growth and development. Natural CKs are derivatives of adenine containing an isoprenoid or aromatic moiety at the N^6 position. Typical representatives of isoprenoid CKs are N^6-(2-isopentenyl)adenine (iP) and its hydroxylated forms

zeatin (Z) and dihydrozeatin (DHZ). Zeatin occurs in two isomers, *cis*Z and *trans*Z, referring to the position of the terminal hydroxyl group on the isoprenoid side chain.

Compelling evidence from several studies has shown that CK signals in plants are able to delay leaf senescence (Gan and Amasino, 1995; Kim *et al.*, 2006, and references therein), promote shoot branching (Thimann and Sachs, 1964; Tanaka *et al.*, 2006), and specifically affect plant shoot and root growth (Werner *et al.*, 2001, 2003). In addition, CKs mediate the signalling of the availability of inorganic nitrogen to the roots (Miyawaki *et al.*, 2004), seed gemination, and responses to pathogens (Mok, 1994).

Most of the physiological activity of zeatin as a free hormone has been attributed for years to *trans*Z, while typically *cis*Z has been considered as an inactive or weakly active form of CK. These conclusions were based on data from various bioassays (Schmitz *et al.*, 1972; Kaminek *et al.*, 1987). *cis*Z has been reported to be an important component of certain tRNAs localized as a modified adenosine residue immediately adjacent to the 3' end of the anticodon of tRNAs that recognize the UUN codon (Skoog and Armstrong, 1970). A strong decrease of free *cis*Z was monitored after knocking down two genes; *AtIPT2* and *AtIPT9*, coding for the CK biosynthesis enzyme isopentenyltransferase (IPT; Miyawaki *et al.*, 2006). They are the only tRNA-specific IPT genes which share substantial similarities with the prokaryotic *miaA* gene catalysing transfer of dimethylallyl diphosphate (DMAPP) to tRNA (Persson and Bjork, 1993; Leung *et al.*, 1997).

Despite the presumed inactivity of *cis*Z as a free hormone, the presence of free *cis*Z-type CKs in plant tissues has been reported repeatedly. Some plant species contain detectable levels of diverse *cis*Z-type CKs, and even occasionally as the predominant group of total CKs. Different *cis*Z-type CKs were identified in a large number of seed plants including both monocots (e.g. Parker *et al.*, 1988; Veach *et al.*, 2003) and dicots (e.g. Nicander *et al.*, 1995; Emery *et al.*, 1998, 2000; Dobrev *et al.*, 2002; Ananieva *et al.*, 2004; Gaudinová *et al.*, 2005; Malkawi *et al.*, 2007; Stirk *et al.*, 2008; Van Staden *et al.*, 2010), as well as in algae (Stirk *et al.*, 2003; Ördög *et al.*, 2004) and mosses (Von Schwartzenberg *et al.*, 2007). Moreover, enzymes catalysing specifically *cis*Z metabolic reactions were reported, namely glucosyltransferases (*cisZOG*1 and *cisZOG*2) in maize (Martin *et al.*, 2001; Veach *et al.*, 2003) and *cis–trans*-isomerase in beans (Bassil *et al.*, 1993). Likewise signal perception by particular histidine kinases can be mediated by *cis*Z and/or its riboside *cis*ZR with similar efficiency to *trans*Z (Spíchal *et al.*, 2004; Yonekura-Sakakibara *et al.*, 2004; Romanov *et al.*, 2006). These findings overall have indicated that *cis*Z derivatives are more prevalent and more relevant to CK biology than previously thought, and probably have unique functions in plant tissues. Moreover, it is speculated that they are synthesized in plant cells in a distinct way(s) compared with their corresponding *trans* isomers (Kasahara *et al.*, 2004). However, a precise role for *cis*Z-type CKs in plants still remains to be elucidated.

The aim of this study was to unravel the distribution of the *cis*Z-type CKs in the plant kingdom, their activity in bioassays, as well as their uptake and metabolism including possible *cis–trans* isomerization in plants. The validity of analytical techniques, excluding spontaneous *cis*Z↔*trans*Z isomerization during the extraction and/or purification procedure, has been evaluated here. These aspects have been surveyed using both monocot and dicot plant models (oat, *Arabidopsis*, and tobacco). Based on the results it is hypothesized that the role of *cis*Z might reside in a delicate regulation of CK response(s) and maintenance of minimal CK activity under growth-limiting conditions.

Materials and methods

Chemicals

All CKs were procured from Olchemim Ltd (Olomouc, Czech Republic); other chemicals were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Radiolabelled CKs [2-^{3}H]iP (specific activity 1200 TBq mol^{-1}), [2-^{3}H]*trans*Z (850 TBq mol^{-1}), and [2-^{3}H] *cis*Z (780 TBq mol^{-1}) were synthesized at the Isotope Laboratory, Institute of Experimental Botany AS CR (Prague, Czech Republic) according to Hanus *et al.* (2000). [$^{13}C_5$]*cis*-zeatin CKs were prepared according to an unpublished method which will be described elsewhere. Other deuterium-labelled CK standards were purchased from Olchemim Ltd.

Plant material

Seeds of plant species chosen for CK screening were obtained from SELGEN Ltd (Pernarec, Czech Republic) and SEMO Ltd (Smržice, Czech Republic). The tissues (in most cases vegetative shoots, otherwise leaves) for analyses of CK content were collected from plants cultivated in optimal greenhouse conditions or from plants growing in the open air and sampled during June and July 2008 in the north-eastern region of the Czech Republic. (A list of the species analysed in this study can be found in Supplementary Table S1 available at *JXB* online.)

Cytokinin analysis

The procedure for CK purification was based on a modification of the method described by Faiss *et al.* (1997). Deuterium-labelled CK internal standards (Olchemim Ltd) were added, each at 1 pmol per sample to check the recovery during purification and to validate the determination (Novák *et al.*, 2008). The samples were purified using a combined cation (SCX-cartridge) and anion (DEAE-Sephadex-C18-cartridge) exchanger and immunoaffinity chromatography (IAC) based on a wide range of specific monoclonal antibodies against CKs (Novák *et al.*, 2003). The metabolic eluates from the IAC columns were evaporated to dryness and dissolved in 20 µl of the mobile phase used for quantitative analysis. The samples were analysed by ultra-performance liquid chromatography (UPLC) (Acquity UPLC™; Waters, Milford, MA, USA) coupled with a Quatro *micro*™ API (Waters, Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray interface. The purified samples were injected onto a C18 reversed-phase column (BEH C18; 1.7 µm; 2.1×50 mm; Waters). The column was eluted with a linear gradient (0 min, 10% B; 0–8 min, 50% B; flow rate of 0.25 ml min^{-1}; column temperature of 40 °C) of 15 mM ammonium formate (pH 4.0, A) and methanol (B). Quantification was obtained by multiple reaction monitoring (MRM) of [M+H]$^+$ and the appropriate product ion. For selective MRM experiments, optimal conditions, dwell time, cone voltage, and collision energy in the collision cell corresponding to an exact diagnostic transition were optimized for each CK

(Novák *et al.*, 2008). Quantification was performed by Masslynx software using a standard isotope dilution method. The ratio of endogenous CK to an appropriate labelled standard was determined and then used to quantify the level of endogenous compounds in the original extract, according to the known quantity of an added internal standard (Novák *et al.*, 2003).

An analysis of profiles and concentrations of individual CKs in plant species with a prevalence of *cis*Z(s) was performed by HPLC-MS/MS. CKs were extracted from plants by methanol/formic acid/water (15/1/4, v/v/v), homogenized in liquid nitrogen, and purified using a dual-mode solid phase extraction method (Dobrev and Kaminek, 2002). CK ribotides were determined as the corresponding ribosides following their dephosphorylation by alkaline phosphatase. The HPLC-MS analysis was performed as described by Dobrev *et al.* (2002) using a Rheos 2000 HPLC gradient pump (Flux Instruments AG, Reinach, BL, Switzerland) and HTS PAL autosamples (CTC Analytics AG, Zwingen, Switzerland) coupled with a Finnigan MAT LCQ-MS^n ion trap mass spectrometer (Finnigan, San Jose, CA, USA) equipped with an electrospray interface. Detection and quantification were carried out using a Finnigan LCQ operated in the positive ion full-scan MS/MS mode using a multilevel calibration graph with ^{2}H-labelled CKs as internal standards. Detection limits were calculated for each compound as 3.3 σ/S (σ is the standard deviation of the response and S is the slope of the calibration curve) and ranged between 0.5 and 1.0 pmol per sample. The results indicate averages of analyses of three independent samples and two HPLC-MS/MS injections for each sample.

Chlorophyll retention bioassay

Oat (*Avena sativa* L. cv. Abel) seeds were soaked for 24 h in aerated distilled water (26 °C, 16 h light /8 h dark) and sown into saturated perlite with a 2-fold concentrated Knop's nutrient solution. Plants were cultivated in a growth chamber (SANYO MLR 350H; Sanyo, Osaka, Japan) with an 18 h light/6 h dark photoperiod (photon flux of 19 995 lx), 20 °C/18 °C, and ~80% relative humidity. The first fully developed leaves were excised from 10-day-old plants when the second leaf just started to develop. The leaf apexes were cut into 7 cm long segments and incubated in test tubes containing 1 ml of CK solutions for 4 d at 26 °C in darkness. Four replicates were prepared from each variant. Chlorophyll was extracted with 80% ethanol according to Kaminek *et al.* (1987); the optical absorbance was measured at 665 nm on a Unicam 5625 spectrometer. CK activities were compared in accordance with EC_{50} values, defined as the concentration at which 50% of the maximum response was recorded. For microscopic studies, the leaves (~2 mm wide) were cut off 1 cm from their apical tips, put into a drop of water on a microscopic slide, covered with a coverslip, and immediately observed under a confocal laser scanning microscope (Leica TCS NT, Heidelberg, Germany) (Schwarzerová *et al.*, 2006). Tested compounds were applied at concentrations of 3.2×10^{-8}, 1.6×10^{-7}, 8×10^{-7}, 4×10^{-6}, 2×10^{-5}, 1×10^{-4}, and 5×10^{-4} M.

Tobacco callus bioassay

CK-dependent tobacco (*Nicotiana tabacum* cv. Wisconsin 38) callus derived from a 4-week-old culture was cultivated on solid MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g l^{-1}), naphthaleneacetic acid (NAA; 1 mg l^{-1}), and the CK to be tested (in a concentration ranging from 0.640 nM to 10 μM). CK activities were expressed as average fresh weight (FW) of tissue per flask after 7 weeks of cultivation in darkness at 23 °C and compared in accordance with the EC_{50} values (see above). Three independent experiments were set up for each CK. The control stock calli were grown on the same medium containing benzyladenine (0.2 mg l^{-1}). The tested compounds were applied in concentrations of 6.4×10^{-10}, 3.2×10^{-9}, 1.6×10^{-8}, 8×10^{-8}, 4×10^{-7}, 2×10^{-6}, and 1×10^{-5} M.

Amaranthus bioassay

A standard bioassay based on the dark induction of betacyanin synthesis in *Amaranthus* cotyledons was carried out as previously described (Holub *et al.*, 1998). The final concentration of solvent [dimethylsulphoxide (DMSO)] in the medium did not exceed 0.2% (v/v). Five replicates were prepared for each CK concentration, and the complete tests were repeated at least three times, the results being compared in accordance with EC_{50} values (see above).

Arabidopsis ARR5:GUS reporter gene assay

This assay was carried out as described in Romanov *et al.* (2002), with slight modifications. For quantitative assays, *ARR5:GUS* seedlings were grown for 2–3 d (22 °C, 16 h light/8 h dark) in six-well plates (Techno Plastic Products Ltd, Zurich, Switzerland) and then *cis*Z, *trans*Z, or control solvent [DMSO, final concentration 0.1% (v/v)] was added to the desired final concentration. The seedlings were then incubated in the dark for 17 h at 22 °C. The results were compared in accordance with EC_{50} values (see above).

Uptake and metabolism of [^{3}H]cisZ and [^{3}H]transZ in tobacco BY-2 *cell culture and in detached oat leaves*

Radiolabelled [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z (2×10^{-9} mol l^{-1} each) uptake and accumulation were measured in CK-autonomus tobacco [*N. tabacum* L. cv. Bright Yellow 2 (BY-2) (Nagata *et al.*, 1992)] cell suspension (0.5 ml aliquots) according to Delbarre *et al.* (1996) as described in Petrášek *et al.* (2003). BY-2 cells were harvested immediately after the addition of CK to the liquid medium (time 0 min) and at 5 min intervals during incubation. The uptake and metabolism of [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z in detached oat leaves were investigated in 8 cm long oat leaf segments incubated in 2.5 ml of water containing 42–47 kBq of radiolabelled *cis*Z and *trans*Z, respectively. The incubation proceeded under continuous light (photon flux of 19 995 lux) at 20 °C for 2, 5, 8, 24, 48, and 96 h. CKs were extracted and purified from 14–16 primary leaf segments (~1 g FW) per sample; for each time interval two independently incubated samples were analysed. Radiolabelled CK metabolites were analysed by HPLC coupled to an online radioactivity detector as described by Gaudinová *et al.* (2005) and identified by comparing their retention times with those of authentic standards.

Computational studies of potential cis–trans *isomerization of zeatin*

Calculations of potential *cis–trans* isomerization of zeatin were performed by a GAUSSIAN 03 program (Frisch *et al.*, 2004) using the DFT Kohn–Sham method with B3LYP exchange and correlation functionals (Becke, 1993) and the 6-31+G(d,p) basis set. Twisted transition state (TS) geometries were obtained using a TS search. The energy differences between the ground state and TS electronic energies corresponded to the activation energy of the isomerization reaction.

Determination of cytokinin oxidase/dehydrogenase activity and substrate specificity

The cytokinin oxidase/dehydrogenase (CKX) from oat and tobacco leaves and BY-2 cells was extracted and partially purified according to Motyka *et al.* (2003) and its activity and substrate specificity were determined by *in vitro* radioisotope assays based on the conversion of 2-^{3}H-labelled CKs ([2-^{3}H]iP, [2-^{3}H]*trans*Z, and [2-^{3}H]*cis*Z) to [^{3}H]adenine. The assay mixture (50 μl final volume) included a 100 mM TAPS-NaOH buffer containing 75 μM 2,6-dichloroindophenol (pH 8.5), 2 μM [2-^{3}H]CK (7.4 TBq mol^{-1} each), and enzyme preparation equivalent to 500 mg tissue FW (corresponding to 0.225 and 0.07 mg protein g^{-1} FW for oat and tobacco leaves, respectively) or 20 mg of tissue FW (corresponding to 0.02 mg protein g^{-1} FW for BY-2 cells). After incubation (1 h and 4 h for oat

and tobacco, respectively) at 37 °C the reaction was terminated and the substrate was separated from the product of the enzyme reaction by HPLC, as described elsewhere (Gaudinová *et al.*, 2005). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

For analysis of the substrate specificity of recombinant CKX enzymes of *Arabidopsis* (AtCKX1, AtCKX2, AtCKX3, AtCKX4, AtCKX5, AtCKX6, and AtCKX7) the CKX genes were cloned into expression vectors pPICZ-A, pPICZ-Aα, and pGAPZα (Invitrogen) with a subcloned N-terminal His-tag. Constitutive expression from pGAPZα clones in the *Pichia pastoris* strain X33 was carried out for 3–5 d in YNB medium supplemented by 2% (w/v) glucose and buffered to pH 6.7 by potassium phosphate. The expression of a pPICZ-A construct (AtCKX7) was maintained in a BMMY medium under the same conditions as above and induced by 1% (v/v) methanol each day. Yeast medium or, for pPICZ-A::AtCKX7, cell extract containing recombinant CKX was concentrated and underwent low-pressure chromatography onto Bio-gel hydroxyapatite (Bio-Rad), octyl-Sepharose CL-4B, and Ni Sepharose HP (Pharmacia) columns as recommended by the supplier [a detailed description of this procedure is given in Morris *et al.* (1999) and Pertry *et al.* (2009)]. The enzyme assay was based on the decolorization of an appropriate electron acceptor—100 μM 2,6-dichlorophenolindophenol (DCIP; Laskey *et al.*, 2003) or 500 μM ferricyanide (FC; Pertry *et al.*, 2009) followed at 420 nm and 600 nm, respectively, within 15 min. The reaction mixture further contained 100 mM McIlvaine buffer, pH 6.5 (DCIP) or pH 6.0 (FC) and 50 μM substrate. The apparent K_m values of CKXs were determined with FC in the same assay mixture as described above and the substrate CKs used in the concentration range of 12.5–100 μM.

Results

Abundance of cis*Z-type cytokinins in the plant kingdom*

In order to define the distribution between *cis*- and *trans*Z-type CKs in land plants, the ratio of these two isomers (as sums of their free bases, ribosides, ribotides, *O*-glucosides, and *N*-glucosides) in fully developed leaves or shoots of >150 representative species of all the main land plant groups in the vegetative stage, including species of bryophytes, lycophytes, and ferns, gymnosperms, and angiosperms, was determined (Fig. 1A; for a detailed list and a phylogenetic tree of all analysed plant species see Supplementary Fig. S1 and Table S1 at *JXB* online). As shown in Fig.1, plants containing *cis*Z derivatives in very high concentrations considerably exceeding those of their *trans* counterparts can be found across the whole evolutionary tree. Representatives of liverworts (*Conocephalum conicum*) and mosses (*Plagiomnium undulatum*), both non-vascular, spore-producing land plants belonging to the bryophytes, were found to contain the *cis*Z type almost exclusively (Supplementary Table S1). The same was true for some of the spore-bearing vascular plants such as ferns, where orders Equisetales, Schizaeales, Salviniales, and mainly Polypodiales *sensu lato* (*s.l.*) were found to include families with strong preferences for *cis*Z forms (Fig. 1B and Supplementary Table S1). Among seed plants, *cis*Zs were dominant in many analysed taxa of monocots (e.g. Liliales, Zingiberales, and Poales; Fig. 1E) but also in dicot plants (e.g. Malphigiales, Fagales, Cornales, Solanales, and Asterales; Fig. 1F), as described in more detail below and in Supplementary Table S1. On the other hand, gymnosperms and ancestral angiosperms, such as Nymphaeales or Magnolliids, seem instead to prefer *trans*Z isoforms (Figs. 1C, D, and Supplementary Table S1).

Next, to depict the contribution of *cis*Z to the whole CK content, the CK spectra were divided into two groups of interest including (i) *cis*Z-type CKs and (ii) non-*cis*Z-type CKs (represented by *trans*Z, iP, and DHZ with their derivatives). In agreement with the structures and physiological activities, both *cis*Z- and non-*cis*Z-type CKs were classified into four functionally different groups, comprised as described in Fig. 2. The total amount of CKs among all tested species varied between ~0.7 pmol g^{-1} FW and 1378 pmol g^{-1} FW. Monocot plants belonging to the families Zingiberaceae (*Elettaria cardamomum*), Musaceae (*Musa acuminatea*), and Liliaceae (*Lilium* cv. *Elodie*, *Lilium martagon*) were found to contain *cis*Z derivatives in concentrations representing >50% of the whole CK content (Fig. 2, Supplementary Table S1). In particular, in leaves of plants of the Poaceae family (*Zea mays*, *Avena sativa*, *Triticum aestivum*, *Dactylis glomerata*, *Agropyron repens*, and *Phragmites australis*) *cis*Z derivatives represented the major CKs (Fig. 2, Supplementary Table S1). The most abundant CK metabolites detected in these genera were *cis*Z-*O*-glucoside (*cis*Z*O*G) and its riboside (*cis*ZR*O*G), representing altogether >90% of the total CKs. In dicot plants, for instance, a similar CK profile with a clear predominance of *cis*Z*O*G and *cis*ZR*O*G was found in *Manihot* sp. (Euphorbiaceae) leaves (Fig. 2). Relatively high levels of *cis*Z-type CKs (exceeding 65% of the total CKs) were also detected in leaves of *N. tabacum*, however, with *cis*Z-N^7-glucoside (*cis*Z7G) as a prevailing CK compound. All CK-type levels of bioactive CK bases and ribosides as well as CK ribotides were rather low in all tested plants. Irrespective of the plant material the prevailing CK forms were *N*- or *O*-glucosides. It can be concluded that *cis*Z-type CKs were detected in all tested plants, but a parallel with evolutionary complexity was not confirmed and they are more likely to be connected by means of a reproductive strategy.

Levels of cis*- and* trans*-zeatins differ during ontogenesis of* Arabidopsis

Further attention was focused on the distribution of the metabolites of the *cis*- and *trans*Z isoforms (free bases, ribosides, ribotides, *O*-glucosides, and *N*-glucosides) throughout plant ontogenesis using *Arabidopsis* as a model plant. It was found that levels of *trans*- and *cis*Zs change dramatically throughout the *Arabidopsis* life span. The ratio between *cis*- and *trans*Z-type CKs is shown in Fig. 3A. Dry seed *cis*Zs were the major CKs, comprising ~70% of the total CK content, with *cis*ZR and *cis*ZRMP as dominant metabolites. No significant changes were observed from the initial observation after the first 24 h of imbibition (Fig. 3A). The situation changed noticeably in the first days of seedling development. On the sixth day after germination (DAG), the level of *cis*Zs decreased dramatically (13%) and

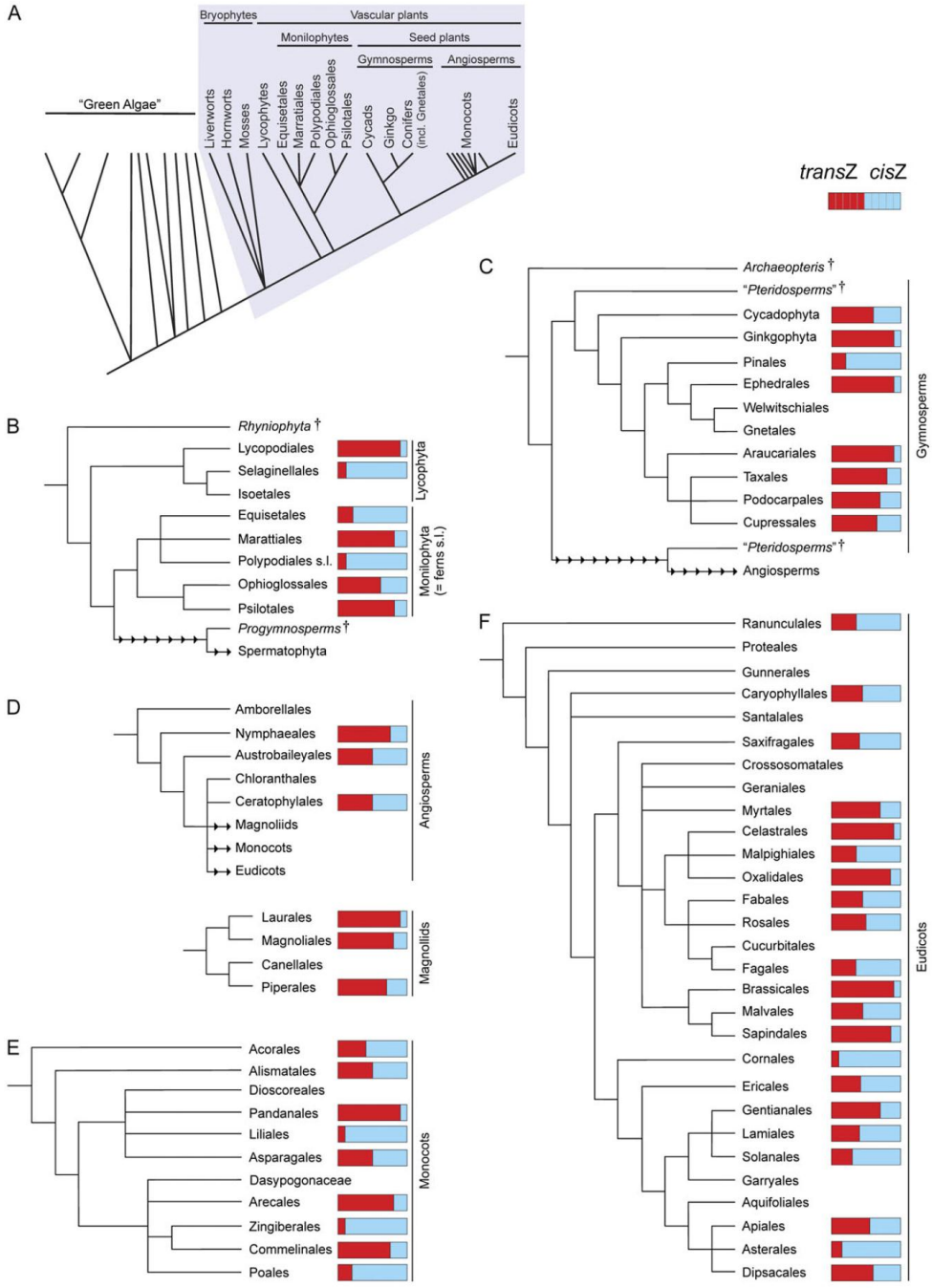

Fig. 1 Distribution between *trans*- and *cis*Z-type CKs throughout the evolution of the plant kingdom. (A) A simplified evolutionary tree of green plants with highlighted main extant plant groups analysed for the ratio of *trans*Z- and *cis*Z-type cytokinins (as the sums of their free bases, ribosides, ribotides, *O*-glucosides, and *N*-glucosides) in this work. (B–F) Phylogenetic relationships and ratios of *trans*Z- and *cis*Z-type cytokinins in the main groups of vascular plants, i.e. Lycophyta and Ferns *s.l.* (B), Gymnosperms (C), Angiosperms and Magnolliids (D), Monocots (E), and Eudicots (F). For a detailed list and phylogenetic tree of all analysed plant species see Supplementary Fig. S1 and Table S1 at *JXB* online. Phylogenetic trees were compiled and modified according to Simpson (2006). Taxa marked with † and written in italics are extinct.

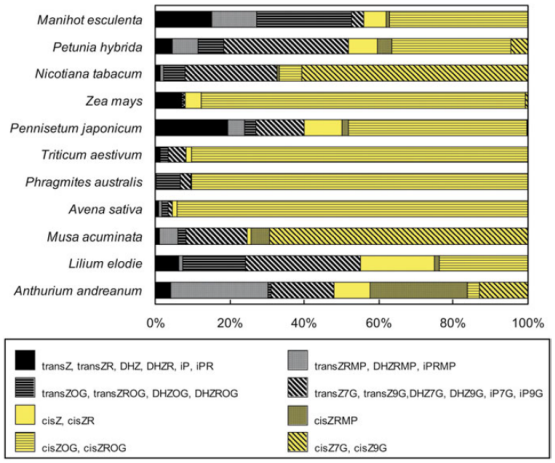

Fig. 2 Proportions of CK groups in leaves of various seed plants and contribution of *cis*Z to the whole CK content. Plant species are arranged from the oldest to the youngest in evolutionary terms (from the bottom to the top); the CK derivatives are divided into eight groups of interest according to structure and activity. Non-*cis*Z-type CKs are shown by black and white bars and *cis*Z-type CKs are shown by black and yellow bars.

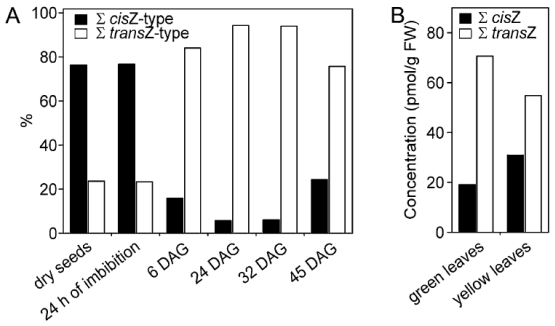

Fig. 3 The ratio between *trans*Z- and *cis*Z-type cytokinins in aerial parts throughout the ontogenesis of *Arabidopsis*. (A) Changes in the *trans*Z- and *cis*Z-type cytokinins ratio during the *Arabidopsis* lifespan from seed to late senescence. (B) Comparison of *trans*Z- and *cis*Z-type cytokinins levels in the youngest green and oldest senescent rosette leaves of 65-day-old *Arabidopsis*.

the major CKs became *trans*Zs (84%; Fig. 3A). The dominance of *trans*Z-type CKs was even more pronounced during the vegetative stage (94%; 24 DAG, 32 DAG), whereas *cis*Z-type CKs were represented by only 5% of the whole CK content (Fig. 3A). During early senescence (45 DAG) the balance shifted towards *cis*Zs (21%; Fig. 3A). The partial rearrangement of the *cis*- and *trans*Z-type CK levels during senescence was also confirmed by analysis of CK content in the youngest green and oldest senescent rosette leaves of 65-day-old *Arabidopsis* plants. In comparison with the youngest green leaves, the abundance of *cis*Zs in the old leaves was 1.6 times higher (Fig. 3B), with the highest increase being in the concentration of *cis*ZR (6.5-fold). The level of *trans*Zs decreased 1.3-fold (Fig. 3B). In the senescent leaves, *cis*Zs represented almost 30% of the total CK content. It seems that the proportion of *cis*Z-type CKs changes during *Arabidopsis* ontogenesis and increases in organs which have completed growth.

Natural cis–trans*Z isomerization without a catalyst is not possible*

In order to exclude the occurrence of *cis*Z in plant samples as an artefact formed during extraction and purification procedures, a computational analysis excluding such a possibility was performed as a part of this study. Alkene *cis*↔*trans* isomerization is generally a photochemical reaction involving excitation of the electron from π to π* orbital. This excitation requires energy of ~5–6 eV, corresponding to the UV region (~230 nm; Foo *et al.*, 1974). An alternative thermal pathway of isomerization has been studied, particularly for the so-called push–pull alkenes (Matus *et al.*, 2003). Such alkenes possess an electron donor substituent on one side of the double bond and an electron acceptor on the other, which results in weakening of the double bond. For extreme cases, the barrier for rotation around the double bond was shown to be as low as 7 kcal mol^{-1}. For the present computational studies, the zeatin molecule was considered as a substituted alkene lacking a purine moiety. The model system (*2E*)-4-amino-2-methyl-but-2-en-1-ol) is depicted in Supplementary Fig. S3 at *JXB* online. The activation energy for thermal isomerization was found to be 58 kcal mol^{-1}, which is comparable with an unsubstituted double bond and corresponds to a reaction rate order of 10^{-28} mol s^{-1} for a monomolecular reaction. As neither a photochemical nor a thermal pathway is accessible under standard laboratory conditions, *cis*Z↔*trans*Z isomerization during the isolation procedure can therefore be excluded without the presence of a catalyst. Conversion catalysed by intensive cool white fluorescent light in an aqueous solution was observed to prefer a *cis* to a *trans* course of isomerization (Bassil *et al.*, 1993) with the ratio balance at 13%. However, the influence of such conditions on the extraction or purification process can be excluded since both zeatin standards, which are exposed to the same light and solvent conditions as samples, are usually checked for stability before the HPLC/MS analysis.

Since zeatins yield identical parent $[M+H]^+$ ions and basic fragment peaks under electrospray mass spectrometry conditions, it was essential to ensure definite separation of the two isomers in order to obtain accurate estimates of their endogenous levels. The analyses without and/or with imperfect separation and internal standardization used sometimes to increase the sample throughput (Prinsen *et al.* 1995) would inevitably cause an overestimation of endogenous concentrations. For this reason, the ring-labelled $[^{13}C_5]$*cis*Z, having a different fragmentation pathway (*cis*Z: 225→141) from a classical side chain-labelled $[^{2}H_5]$*trans*Z (*trans*Z: 225→136), was synthesized. The eluting ions were

measured by MRM of $[M+H]^+$ in a positive ion mode and of the appropriate product ion. The addition of the labelled zeatins to different extracts further facilitated their quantification, also giving better resolution in natural samples as well as a measure of possible *cis*↔*trans* interconversion. In no samples analysed in this study was *cis*↔*trans* Z isomerization observed.

Biological activity of cis- *and* trans-*zeatin in different cytokinin biotests*

Most of the physiological activity of zeatin has been attributed for years to *trans*Z, while *cis*Z has been considered mainly as a weakly active CK. For this reason the biological activity of *cis*Z and *trans*Z metabolites (for free bases, see Fig. 4; the results for ribosides and *O*-glucosides are given in the Supplementary data at *JXB* online) were compared in various CK bioassays.

In the oat leaf senescence assay all tested *trans*Z-type CKs suppressed chlorophyll degradation efficiently (Fig. 4A, Supplementary Fig. S2A, B). All *cis*Z-type CKs were also effective in delaying dark-induced senescence but with lower efficiency (between 5- and 50-fold when compared in accordance with EC_{50} values) than their corresponding *trans* counterparts (Fig. 4A, Supplementary Fig. S2A, B). The most pronounced chlorophyll retention was observed after *cis*ZR application at a concentration 500 μM, when ~92% of the maximum responses induced by corresponding *trans* isomers were reached. Corresponding results demonstrating the activities of *cis*Z-type CKs and their *trans* counterparts (free bases, ribosides, and *O*-glucosides) in delaying dark-induced chlorophyll degradation were also obtained with excised wheat leaves in the same assay and with maize leaf segments in a drop bioassay (data not shown). To support the chlorophyll retention data for inhibition of plastid disintegration in oat leaves treated with *cis*- or *trans*Z, leaf specimens were monitored with a laser scanning microscope and the images are depicted in Fig. 4B.

Biological activities of *cis*Z- and *trans*Z-type CKs in promoting tobacco callus growth are demonstrated in Fig. 4C, and Supplementary Fig. S2C and D at *JXB* online. All tested *trans* isomers considerably enhanced tobacco cell division. The lowest effective concentrations were *trans*Z < *trans*ZR < *trans*ZOG, with the maximum stimulatory responses at concentrations ~25 times higher. All tested *cis*Z-type CKs were remarkably effective in promoting tobacco callus growth, displaying activities in the order *cis*ZR > *cis*Z > *cis*ZOG (based on EC_{50} values; Fig. 4C, Supplementary Fig. S2C, D). The most pronounced biological response on callus growth was recorded for *cis*ZR at 400 nM, which represented >90% of the highest activity observed for its *trans* counterpart. In general, the *trans*-Z:*cis*Z activity ratio of assayed CK derivatives varied between ~3 and 27 (in accordance with EC_{50} values; Fig. 4C). These data suggest a possible interchangeability of *trans*Z-type CKs by high concentrations of *cis*Z-type CKs at least in terms of maintaining cell division.

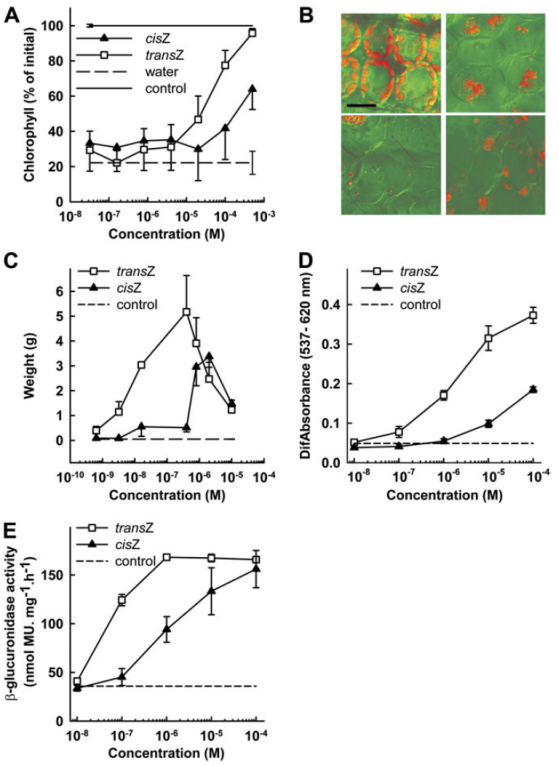

Fig. 4 Comparison of the activity of *trans*Z and *cis*Z in different CK bioassays and their induction of CK signalling. (A) Effect on retention of chlorophyll in senescing oat leaves. Values are expressed as a percentage of the initial chlorophyll content of fresh leaves before incubation. (B) Images of oat leaf mesophyll cells and their chloroplasts (original red autofluorescence). From the upper left corner to the right: control (fresh oat leaves before the experiment), and leaves treated with *cis*Z, water, or *trans*Z after 10 d of cultivation in the dark. The bar represents 20 μm. (C) Effect on fresh weight yield of tobacco cytokinin-dependent W-38 callus tissue. (D) Effect on dark induction of betacyanin synthesis in *Amaranthus* cotyledon–hypocotyl explants. (E) Induction of expression of the *ARR5:GUS* cytokinin reporter in transgenic *Arabidopsis* seedlings. Error bars show the SD (n=4 for A, C, n=5 for D, and n=2 for E); entire tests were repeated at least twice. Dashed lines indicate control treatment without added cytokinin.

In the *Amaranthus* bioassay, *trans*Z was again the more active isomer in dark induction of betacyanin synthesis and reached an EC_{50} value of 1.8 μM, whereas the EC_{50} value for *cis*-zeatin was >100 μM (Fig. 4D). At this concentration *cis*Z reached only 44% of *trans*Z maximal activity.

The competency of *cis*Z to activate CK signalling components subsequent to CK receptor interaction was investigated by means of the *ARR5:GUS* reporter gene response, which is known to be rapidly up-regulated by CK (D'Agostino *et al.*, 2000; Romanov *et al.*, 2002). Data presented in Fig. 4E show that both *trans*- and *cis*Z were able to induce the level of ARR5:GUS in a dose-dependent

manner, although with different effectivity. While *trans*Z was a potent inducer of ARR5:GUS expression, reaching a submicromolar EC_{50} value of 0.07 μM, *cis*Z reached an EC_{50} value of 1.2 μM, indicating the ability of *cis*Z to induce a CK response effectively at the molecular level. Apparently a high abundance of *cis*Z is needed for biological activity in all CK bioassays.

Uptake and accumulation of [^{3}H]cisZ and [^{3}H]transZ by tobacco BY-2 cells

To acquire information about the uptake and accumulation of *cis*- and *trans*Z-type CKs by plant cells, radiolabelled [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z were added to a liquid medium of the tobacco BY-2 suspension culture. The results showed progressive accumulation of both *cis*Z and *trans*Z in cultured tobacco cells with no preference for either of the isomers (Fig. 5). Most of the radioactivity of *cis*Z as well as *trans*Z (up to 80–90% of the total) was taken up early, within 15 min. It would seem that both zeatin isomers entered cells relatively rapidly and in the same or a similar way. Transport does not therefore seem to be a regulatory point in *cis*Z activity.

Short-term metabolism of [^{3}H]cisZ and [^{3}H]transZ in tobacco BY-2 suspension culture

The fate of radiolabelled zeatin metabolites in tobacco BY-2 cells was determined at four time points during the first 15 min of the [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z accumulation experiment. Radiolabelled *cis*Z was metabolized in BY-2 cells, with only a few metabolites detected. A gradual decrease of [^{3}H]*cis*Z (from 72% to 31% of total radioactivity within 15 min incubation) was accompanied by a corresponding increase in radioactivity associated mostly with *cis*ZRMP and to a lesser extent with *cis*ZR. Accumulation of adenine indicated degradation of *cis*Z by CKX (Fig. 6A). Adenosine was detected only in minute amounts. Compared wiht *cis*Z, the application of [^{3}H]*trans*Z to BY-2 cells led to the detection of more CK forms including *N*- and *O*-glucosides. Similarly to *cis*Z, the prevailing metabolites of [^{3}H]*trans*Z were its corresponding ribotide (*trans*ZRMP), riboside (*trans*ZR), and adenine (Fig. 6B). In addition *trans*Z-N^7-glucosides and *trans*Z-*O*-glucosides were found in cells immediately after the addition of [^{3}H]*trans*Z to the media; however, their contribution to the total pool of metabolites remained low. The metabolic fate of *cis*Z differs from that of *trans*Z in terms of slower metabolic changes generating different spectra of metabolites. Interestingly, no *cis*Z↔*trans*Z isomerization was observed during the feeding experiments in BY-2 cells.

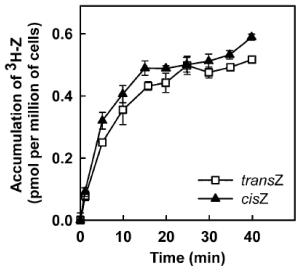

Fig. 5 Uptake of [^{3}H]*cis*Z and [^{3}H]*trans*Z by tobacco BY-2 suspension-cultured cells. Error bars represent the SD.

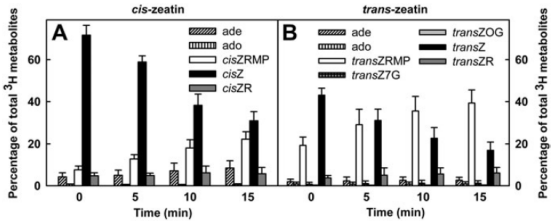

Fig. 6 Metabolism of [^{3}H]*cis*Z and [^{3}H]*trans*Z in tobacco BY-2 suspension-cultured cells. The bars represent the distribution of radioactivity associated with individual CK metabolites 5, 10, and 15 min after application of [^{3}H]*cis*Z (A) or [^{3}H]*trans*Z (B); the values are expressed as a percentage of the total extracted radioactivity taken up by cells. Error bars represent the SD (Ade, adenine; Ado, adenosine; *cis*Z, *cis*-zeatin; *cis*ZR, *cis*-zeatin riboside; *cis*ZRMP, *cis*-zeatin riboside-5′-monophosphate; *trans*Z, *trans*-zeatin; *trans*ZR, *trans*-zeatin riboside; transZRMP, *trans*-zeatin riboside-5′-monophosphate; *trans*Z7G, *trans*-zeatin 7-glucoside; *trans*-ZOG, *trans*-zeatin *O*-glucoside).

Metabolism of [^{3}H]cisZ and [^{3}H]transZ in detached oat leaves

Having in view obtaining information about long-term interconversions of *cis*- and *trans*Z-type CKs in plants, the uptake and metabolic fate of [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z were investigated in detached oat leaves. Most of [^{3}H]*cis*Z was metabolized to adenine (up to 50% within 8 h) and to a lesser extent but progressively to adenosine (~8% after 96 h; Fig. 7A) indicating degradation of *cis*Z by CKX. In accordance with the prominent occurrence of endogenous *cis*Z*O*G in oat leaves (Fig. 2), radiolabelled *cis*Z*O*G was detected as an abundant [^{3}H]*cis*Z metabolite (Fig. 7A). During the whole incubation period, a significant proportion (12% of total metabolites) of [^{3}H]*cis*Z was retained in an unmetabolized form. The degradation products adenine and adenosine were also found as prominent metabolites of [2-^{3}H]*trans*Z in oat leaves (Fig. 7B). In contrast to *cis*Z, the application of [^{3}H]*trans*Z to oat leaves led to the detection of corresponding N^7- and N^9-glucosides (Fig. 7B). Neither [^{3}H]*cis*Z nor [^{3}H]*trans*Z was found to be a subject of *cis*Z↔*trans*Z isomerization. Similarly, a variation in metabolism between [^{3}H]*cis*Z and [^{3}H]*trans*Z, with more metabolites detected after [^{3}H]*trans*Z treatment, was observed in detached tobacco cv. Samsun leaves (data not shown). Also these plant materials confirmed a distinct pattern for *cis*Z and *trans*Z metabolism and slower accumulation of *cis*Z metabolites.

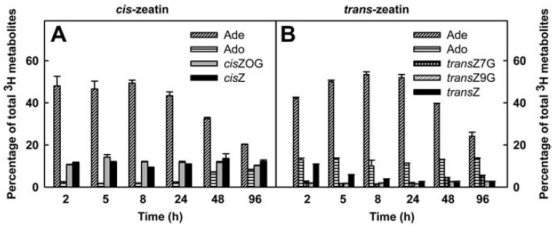

Fig. 7 Metabolism of [^{3}H]*cis*Z and [^{3}H]*trans*Z in oat leaf segments. The bars represent the distribution of radioactivity associated with individual CK metabolites 2, 5, 8, 24, 48, and 96 h after application of [^{3}H]*cis*Z (A) or [^{3}H]*trans*Z (B); the values are expressed as a percentage of the total radioactivity taken up by excised leaves. (Ade, adenine; Ado, adenosine; *cis*Z, *cis*-zeatin; *cis*ZOG, *cis*-zeatin *O*-glucoside; *trans*Z, *trans*-zeatin; *trans*Z7G, *trans*-zeatin 7-glucoside; *trans*Z9G, *trans*-zeatin 9-glucoside).

CKX activity and substrate specificity toward zeatin isoforms

With respect to the intense *in vivo* formation of adenine and adenosine as products of *cis*Z and *trans*Z metabolism in tobacco BY-2 cells and oat leaves (Figs 6, 7), degradation of [2-^{3}H]*cis*Z, [2-^{3}H]*trans*Z, and [2-^{3}H]iP by CKX from crude extracts of two plant materials was determined. The *in vitro* enzymatic studies revealed the order of preference of potential CKX substrates iP > *cis*Z > *trans*Z for tobacco BY-2 cell and cv. Samsun leaf, and iP > *cis*Z = *trans*Z for oat leaf enzymes (Table 1), which demonstrate their higher or identical affinities for the *cis* isomer compared with the *trans* isomer.

From this point on, the influence of the cellular localization and expression pattern of CKX isoforms on their capability to degrade *cis*Z and *trans*Z was studied using purified recombinant CKXs of *Arabidopsis*. The activity of CKX enzymes toward iP, *trans*Z, *cis*Z, and their ribosides was estimated as the initial rates of decolorization of the electron acceptors DCIP or FC (Table 2). Whereas reaction rates of iP and *trans*Z were not significantly varied for any of the characterized enzymes, the capability to degrade *cis*Z and its riboside differed with respect to an individual isoform. Two major apoplastic CKXs of *Arabidopsis*, AtCKX2 and AtCKX4, with relevant expression in different tissues throughout plant ontogenesis, showed only limited degradation of *cis*Z (Table 2, Supplementary Table S2 at *JXB* online). *cis*Z was almost resistant to vacuolar-targeted AtCKX3, showing considerable expression during flowering (Table 2, Supplementary Table S2). Interestingly, the second *Arabidopsis* isoform targeted to vacuoles, AtCKX1, deactivated *cis*Z very effectively (Table 2). As both vacuolar CKXs showed different patterns of expression, selective accumulation of *cis*Z derivatives in vacuoles might have a temporal or tissue-specific character. The CKX isoform AtCKX7 with cytosolic localization was found to degrade *cis*Z as a preferred substrate in comparison with other isoprenoid CKs (Table 2).

Table 1. *Substrate specificity of crude extracts of CKX enzymes toward iP,* trans*Z, and* cis*Z*

The CKX activity was determined using [2-^{3}H]N^6-(2-isopentenyl)adenine, [2-^{3}H]*trans*-zeatin, and [2-^{3}H]*cis*-zeatin, 2 μM each, as substrates. Enzyme activity was measured in 100 mM TAPS-NaOH buffer containing 75 μM 2,6-dichloroindophenol at pH 8.5 and is expressed as relative rates toward iP degradation (100%).

	iP	*trans*Z	*cis*Z
Oat leaves	100	19	21
Tobacco leaves	100	8	37
BY-2 cells	100	16	62

Discussion

*cis*Z has usually been thought of as being a CK with low activity. However, a number of reports previously demonstrated a high abundance of *cis*Z and/or its derivatives in algae, mosses, and various seed plants. The present comprehensive screen throughout the evolution of land plants endorses the hypothesis that *cis*Z-type CKs occur in the plant kingdom ubiquitously. Surprisingly *cis*Z and *trans*Z isomer ratios do not seem to have a direct connection to evolutionary relationships among species (Figs 1, 2, Supplementary Table S1 at *JXB* online). It is known that wheat and other cereals have adopted a strategy of switching from vegetative to generative reproductive growth after pollination to ensure seed filling, and resulting in regulated shoot withering (Sykorova *et al.*, 2008). It is supposed that the prevalence of *cis*Z derivatives especially in Poaceae might be related to this phenomenon and emanate from ontogeny.

It is shown here that the proportions of isomers can differ significantly during the life span of *Arabidopsis*, with particular stages where *cis*Zs represent the major CKs (Fig. 3A, B). Seeds, imbibed seeds, and senescent leaves containing high levels of *cis*Z CKs are characterized by growth cessation but preserved capability for physiological processes. It is assumed that CKs with low activity such as *cis*Zs might be responsible for the maintenance of basal CK activity necessary for plant survival and subsequent recovery. High levels of *cis*Z-type CKs were also found in *Mercurialis* associated with the induction of male sterility (Louis *et al.*, 1990). Recently, rapid accumulation of *cis*ZRMP has been found in maize roots exposed to salinity stress, while *trans*Z levels remained nearly unchanged (Vyroubalová *et al.*, 2009). Remarkable increases in *cis*Z derivatives were observed in plants exposed to severe drought (Havlová *et al.*, 2008), heat (Dobrá *et al.* 2010), or biotic stress (Pertry *et al.*, 2009), and after administration of inhibitors limiting growth (Blagoeva *et al.*, 2003, 2004). Lower seed dormancy of annual rye grass was accompanied by higher levels of *cis*Z (Goggin *et al.*, 2010). Also a reduction of *cis*ZR levels in buds of chickpea was recorded after decapitation (Mader *et al.*, 2003) and probably facilitates release of the bud from dormancy. Obviously all these states are associated with growth-limiting conditions resulting from internal or external cues, and *cis*Z CKs may have a role on these occasions.

Table 2. *Substrate specificity of CKX enzymes toward iP,* trans*Z,* cis*Z and its ribosides, and the spatial and temporal pattern of expression of CKX genes*

Enzyme activity was determined as the initial rate of 2,6-dichlorophenolindophenol decolorization at pH 6.5 and is given as relative rates toward iP degradation (100%). Each number represents the mean value of three replicates where the standard error does not exceed 10%. Specificity of expression was derived from Genevestigator (Zimmermann *et al.*, 2004).

	iP	iPR	*trans*Z	*trans*ZR	*cis*Z	*cis*ZR	Significant expression during development	Organs with the highest expression
Apoplastic								
AtCKX2	100	15	78	7	2.5	0.2	Senescence	Endosperm
AtCKX4	100	13	89	11	3.7	1.0	Rosette development, onset of flowering	Root cap, leaf
AtCKX5	100	72	68	61	68	46	Germination, flowering, senescence	Testa, old leaf
AtCKX6	100	98	13	11	37	22	Bolting	Hypocotyl, shoot apex
Vacuolar								
AtCKX1	100	96	72	58	116	72	Seedlings, bolting	Endosperm, hypocotyl
AtCKX3[a]	100	128	20	32	0.1	0.5	Late flowering	Sepals
Cytosolic								
AtCKX7	100	45	30	5	120	33	No data available[b]	No data available[b]

[a] Ferricyanide decolorization at pH 6.0 was used instead of DCIP which is not an efficient electron acceptor for this enzyme.
[b] Not present on the ATH1 22K Arabidopsis Affymetrix GeneChip.

The occurrence of *cis*Z as an artefact formed during the extraction procedure was proposed in a few studies (Tay *et al.*, 1986) and posed a challenging question concerning *cis*Z stability (Bassil *et al.*, 1993). The present computational analysis showed clearly that the energy necessary for *cis–trans* isomerization between *cis*Z and *trans*Z and vice versa is too high to allow spontaneous transition. The addition of [$^{13}C_5$]*cis*Z and [$^{2}H_5$]*trans*Z, which have a different fragmentation pathway, to the analysed extracts proved the absence of *cis*Z↔*trans*Z isomerization. Therefore, uncontrollable *cis*Z–*trans*Z isomerization during the sample extraction and purification by the procedure used in this study can reliably be excluded.

Part of the problem concerning diverging experimental data relating to the biological activity of *cis*Z could arise from the synthesis and purity of distinct *cis*Z preparations, namely from their possible contamination with *cis*-isozeatin (Leonard *et al.*, 1971). It was found here that preparations of *cis*Z obtained from different sources often exhibited very different biological activities (results not shown).

The data further imply that there is no difference in uptake and accumulation of *cis*Z and *trans*Z by tobacco BY-2 cells that could lead to the differential activity of isomers. Both zeatin isomers are taken up by cells rapidly and in the same or a similar way (Fig. 5). It is, however, questionable whether they enter cells by simple diffusion or via specific transporters. The possible involvement of purine permeases (PUPs) AtPUP1 and AtPUP2 (Bürkle *et al.*, 2003) suggests a plausible function for PUPs in the transport of CK bases including *cis*Z. Transport of CK ribosides can be mediated by a group of equilibrative nucleoside transporters (ENTs) (Hirose *et al.*, 2005), but elucidating the role of other transporters in active transport of zeatin-type CKs requires further investigation. However, transport seems not to be crucial for the difference in zeatin bioactivity. It should be stressed that the absence of a difference in uptake and accumulation of the two zeatin isomers in cultured tobacco cells does not provide appropriate information about the up- and downloading as well as translocation of different CKs including the *cis*- and *trans*Zs in plants. Significant differences in levels and dynamics of the two forms of zeatins in the xylem and phloem channels during development of white lupine seeds indicate their functioning in CK supply and control of development of these organs (Emery *et al.*, 2000). However, speciation of the involvement of xylem and phloem sap CKs in control of CK levels in plant organs is complicated by expression of IPTs in cells associated with both xylem and phloem channels (Miyawaki *et al.*, 2004).

The most important conclusion stemming from metabolic studies consists of unobserved conversion of the labelled *cis* into the *trans* isomer and vice versa regardless of whether short or long feeding experiments were studied (Figs 6, 7). It briefly questions the role of *cis–trans* isomerase (Bassil *et al.*, 1993), but it needs to be fully understood that the enzyme may be activated only under certain circumstances and thus the conditions or type of material used herein might be insufficient to provoke its activity. In general, the metabolic fate of both isomers differs. The two isomers can be *N*- or *O*-glucosylated in a species-specific manner (Figs 2, 6, 7) and degraded by specific CKX enzymes (Table 2). It can be concluded that *cis*Z plays a role in metabolism separately from *trans*Z. *cis*Z interaction with an AHK3 receptor (Spíchal *et al.*, 2004; Romanov *et al.*, 2006) and expression of ARR5 in *Arabidopsis* confirms a relevant CK signalling cascade evoked by *cis*Z. Recent data indicate that *cis*Z might influence—probably by means of competition with other more active CKs on the AtAHK3 receptor—the transport of auxin (Saleem *et al.* 2010) and thus could elicit responses resulting in the preservation of only essential physiological processes.

Biological activity of *cis*Z and its derivatives was demonstrated in tobacco callus, *Amaranthus*, and oat chlorophyll retention bioassays (Fig. 4). CK activity of *cis*Zs is generally lower than that of the corresponding *trans* isomers. The exception is *cis*ZR as the most effective tested CK among

all tested *cis*Z derivatives and only slightly less active than its *trans* counterpart (Supplementary Fig. S2 at *JXB* online). Data demonstrating high bioactivities of CK ribosides compared with other CK forms were also reported in other studies (Kamínek *et al.*, 1979, 1987; Tarkowská *et al.*, 2003; Doležal *et al.*, 2007). High *cis*ZR activity in bioassays might be due to the more efficient transport of CK ribosides and/or in lower degradation by CKX (Sakakibara, 2006). It also hints at a preference for *cis*ZR rather than *cis*Z in the mediation of the extracellular signal transduction to influence physiological effect(s). It is known that potato hinders vegetative growth of its aerial part during tuberization (Fischer *et al.*, 2008). Therefore, the *cis*ZR increase documented in above-ground potato tissue preceding the onset of tuberization (Mauk and Langille, 1978) might serve in preservation of vital but not growing green tissue, ensuring allocation of more nutrients for tuber formation to stolons. Also *cis*ZR accumulation at the end of embryogenesis in developing pea embryos (Quesnelle and Emery, 2007) might be associated with cessation of growth of the embryo. Additionally *cis*ZR has a high resistance against degradation by AtCKX2, the CKX isoform that is expressed during senescence.

It is suggested that the *cis*Z function might consist of maintaining a minimal level of CK response accompanied by restriction of shoot growth to retain plant fitness while another more vital process requiring energy would be preserved or completed. Therefore, the *cis*Z isomer and/or its derivatives may be relevant under growth-limiting conditions associated with a developmental programme or external signals leading to plant optimal survival via reduction but not complete cessation of CK signalling. This hypothesis does not, however, exclude (a) potential role(s) of *cis*Zs in regulation of other physiological processes in plants.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Phylogenetic relationships of vascular plants (compiled and modified according to Simpson, 2006). Taxa marked with † and in italics are extinct. Taxa in red were analysed in this study.

Figure S2. Comparison of activity of ZR and Z*O*G isomers in different CK bioassays. Effect on retention of chlorophyll in senescing oat leaves of ZR (A) and Z*O*G (B). Values are expressed as a percentage of the initial chlorophyll content of fresh leaves before incubation. Effect of ZR (C) and Z*O*G (D) on fresh weight yield of tobacco cytokinin-dependent W-38 callus tissue.

Figure S3. A molecule of *cis*-zeatin (A) and a simplified model of *cis*-zeatin used for the computational study (B).

Table S1. Distribution of *cis*- and *trans*Z-type CKs throughout the evolution of the plant kingdom. Complete list of plants analysed for the ratio of *trans*Z- and *cis*Z-type of cytokinins (as sums of their free bases, ribosides, ribotides, *O*-glucosides, and *N*-glucosides). Means of two independent measurements are shown. The standard deviation did not exceeded 10%.

Table S2. Apparent Michaelis constants for *Arabidopsis* CKX2 and CKX3. All values represent the mean concentration of at least three replications.

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References

Ananieva K, Malbeck J, Kamínek M, van Staden J. 2004. Changes in endogenous cytokinin levels in cotyledons of Cucurbita pepo (zucchini) during natural and dark-induced senescence. *Physiologia Plantarum* **122,** 133–142.

Bassil NV, Mok DWS, Mok MC. 1993. Partial purification of a *cis–trans*-isomerase of zeatin from immature seed of Phaseolus vulgaris L. *Plant Physiology* **102,** 867–872.

Becke AD. 1993. Density-functional thermochemistry. III. The role of exact exchange. *Journal of Chemical Physics* **98,** 5648–5652.

Blagoeva E, Dobrev PI, Malbeck J, Motyka V, Strnad M, Hanuš J, Vaňková R. 2004. Cytokinin N-glucosylation inhibitors suppress deactivation of exogenous cytokinins in radish, but their effect on active endogenous cytokinins is counteracted by other regulatory mechanisms. *Physiologia Plantarum* **121,** 215–222.

Blagoeva E, Malbeck J, Gaudinová A, Vaněk T, Vaňková R. 2003. Cyclin-dependent kinase inhibitor, roscovitine, in combination with exogenous cytokinin, N^6-benzyladenine, causes increase of cis-cytokinins in immobilized tobacco cells. *Biotechnology Letters* **25,** 469–472.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Bürkle L, Cedzich A, Döpke C, Stransky H, Okumoto S, Gillissen B, Kühn C, Frommer WB. 2003. Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of Arabidopsis. *The Plant Journal* **34,** 13–26.

D'Agostino IB, Deruere J, Kieber JJ. 2000. Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiology* **124,** 1706–1717.

Delbarre A, Muller P, Imhoff V, Guern J. 1996. Comparison of mechanisms controlling uptake and accumulation of 2,4-

dichlorophenoxyacetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. *Planta* **198,** 532–541.

Dobra J, Motyka V, Dobrev P, Malbeck J, Prasil IT, Haisel D, Gaudinova A, Havlova M, Gubis J, Vankova R. 2010. Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content. *Journal of Plant Physiology* **167,** 1360–1370.

Dobrev P, Motyka V, Gaudinová A, Malbeck J, Trávníčková A, Kamínek M, Vaňková R. 2002. Transient accumulation of cis- and trans-zeatin type cytokinins and its relation to cytokinin oxidase activity during cell cycle of synchronized tobacco BY-2 cells. *Plant Physiology and Biochemistry* **40,** 333–337.

Dobrev PI, Kaminek M. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *Journal of Chromatography A* **950,** 21–29.

Doležal K, Popa I, Hauserová E, Spíchal L, Chakrabarty K, Novák O, Kryštof V, Voller J, Holub J, Strnad M. 2007. Preparation, biological activity and endogenous occurrence of N^6-benzyladenosines. *Bioorganic and Medicinal Chemistry* **15,** 3737–3747.

Emery RJN, Leport L, Barton JE, Turner NC, Atkins CA. 1998. Cis-isomers of cytokinins predominate in chickpea seeds throughout their development. *Plant Physiology* **117,** 1515–1523.

Emery RJN, Ma Q, Atkins CA. 2000. The forms and sources of cytokinins in developing white lupine seeds and fruits. *Plant Physiology* **123,** 1593–1604.

Faiss M, Zalubilová J, Strnad M, Schmülling T. 1997. Conditional transgenic expression of the ipt gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. *The Plant Journal* **12,** 401–415.

Fischer L, Lipavska H, Hausman JF, Opatrny Z. 2008. Morphological and molecular characterization of a spontaneously tuberizing potato mutant: an insight into the regulatory mechanisms of tuber induction. *BMC Plant Biology* **8,** 117.

Foo PD, Innes KK. 1974. New experimental tests of existing interpretations of electronic transitions of ethylene. *Journal of Chemical Physics* **60,** 4582–4589.

Frisch MJ, Trucks GW, Schlegel HB, *et al*. 2004. *Gaussian 03*. Wallingford, CT: Gaussian, Inc.

Gan S, Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270,** 1986–1988.

Gaudinová A, Dobrev PI, Šolcová B, Novák O, Strnad M, Friedecký D, Motyka V. 2005. The involvement of cytokinin oxidase/dehydrogenase and zeatin reductase in regulation of cytokinin levels in pea (Pisum sativum L.) leaves. *Journal of Plant Growth Regulation* **24,** 188–200.

Goggin DE, Emery RJN, Powles SB, Steadman KJ. 2010. Initial characterisation of low and high seed dormancy populations of Lolium rigidum produced by repeated selection. *Journal of Plant Physiology* **167,** 1282–1288.

Hanuš J, Siglerová V, Matucha M. 2000. N^6-alkyladenosines and adenines labeled with tritium. *Journal of Labelled Compounds & Radiopharmaceuticals* **43,** 523–531.

Havlová M, Dobrev PI, Motyka V, Štorchová H, Libus J, Dobrá J, Malbeck J, Gaudinová A, Vanková R. 2008. The role of cytokinins in responses to water deficit in tobacco plants over-expressing trans-zeatin O-glucosyltransferase gene under 35S or SAG12 promoters. *Plant, Cell and Environment* **31,** 341–353.

Hirose N, Makita N, Yamaya T, Sakakibara H. 2005. Functional characterization and expression analysis of a gene, OsENT2, encoding an equilibrative nucleoside transporter in rice suggest a function in cytokinin transport. *Plant Physiology* **138,** 196–206.

Holub J, Hanuš J, Hanke DE, Strnad M. 1998. Biological activity of cytokinins derived from ortho- and meta-hydroxybenzyladenine. *Plant Growth Regulation* **26,** 109–115.

Kamínek M, Pačes V, Corse J, Challice JS. 1979. Effect of stereospecific hydroxylation of N^6-(Δ^2-isopentenyl)adenosine on cytokinin activity. *Planta* **145,** 239–243.

Kamínek M, Vaněk T, Motyka V. 1987. Cytokinin activities of N^6-benzyladenosine derivatives hydroxylated on the side-chain phenyl ring. *Journal of Plant Growth Regulation* **6,** 113–120.

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, Kamiya Y, Yamaguchi S, Sakakibara H. 2004. Distinct isoprenoid origins of cis- and trans-zeatin biosyntheses in Arabidopsis. *Journal of Biological Chemistry* **279,** 14049–14054.

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I. 2006. Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **103,** 814–819.

Laskey JG, Patterson P, Bilyeu K, Morris RO. 2003. Rate enhancement of cytokinin oxidase/dehydrogenase using 2,6-dichloroindophenol as an electron acceptor. *Plant Growth Regulation* **40,** 189–196.

Leonard NJ, Playtis AJ, Skoog F, Schmitz RY. 1971. Stereoselective synthesis of *cis*-zeatin. *Journal of the American Chemical Society* **93,** 3056–3058.

Leung HCE, Chen Y, Winkler ME. 1997. Regulation of substrate recognition by the MiaA tRNA prenyltransferase modification enzyme of *Escherichia coli* K-12. *Journal of Biological Chemistry* **272,** 13073–13083.

Louis JP, Augur C, Teller G. 1990. Cytokinins and differentiation process in Mercurialis annua—genetic regulation, relations with auxins, indoleacetic-acid oxidases, and sexual expression patterns. *Plant Physiology* **94,** 1535–1541.

Mader JC, Emery RJN, Turnbull CGN. 2003. Spatial and temporal changes in multiple hormone groups during lateral bud release shortly following apex decapitation of chickpea (Cicer arietinum) seedlings. *Physiologia Plantarum* **119,** 295–308.

Malkawi A, Jensen BL, Langille AR. 2007. Plant hormones isolated from 'Katahdin' potato plant tissues and the influence of photoperiod and temperature on their levels in relation to tuber induction. *Journal of Plant Growth Regulation* **26,** 308–317.

Martin RC, Mok MC, Habben JE, Mok DWS. 2001. A maize cytokinin gene encoding an O-glucosyltransferase specific to

cis-zeatin. *Proceedings of the National Academy of Sciences, USA* **98,** 5922–5926.

Matus MH, Contreras R, Cedillo A, Galvan M. 2003. Wave function instabilities in the *cis–trans* isomerization and singlet–triplet energy gaps in a push–pull compound. *Journal of Chemical Physics* **119,** 4112–4116.

Mauk CS, Langille AR. 1978. Physiology of tuberization in Solanum tuberosum L.: cis-zeatin riboside in potato plant: its identification and changes in endogenous levels as influenced by temperature and photoperiod. *Plant Physiology* **62,** 438–442.

Miyawaki K, Matsumoto-Kitano M, Kakimoto T. 2004. Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. *The Plant Journal* **37,** 128–138.

Miyawaki K, Tarkowski P, Matsumoto-Kitano M, Kato T, Sato S, Tarkowska D, Tabata S, Sandberg G, Kakimoto T. 2006. Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proceedings of the National Academy of Sciences, USA* **103,** 16598–16603.

Mok MC. 1994. *Cytokinins and plant development: an overview*. Boca Raton, FL: CRC Press.

Morris RO, Bilyeu KD, Laskey JG, Cheikh NN. 1999. Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. *Biochemical and Biophysical Research Communications* **255,** 328–333.

Motyka V, Vaňková R, Čapková V, Petrášek J, Kamínek M, Schmülling T. 2003. Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. *Physiologia Plantarum* **117,** 11–21.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Nagata T, Nemoto Y, Hasezawa S. 1992. Tobacco BY-2 cell line as the 'HeLa' cell in the cell biology of higher plants. *International Review of Cytology* **132,** 1–30.

Nicander B, Bjorkman PO, Tillberg E. 1995. Identification of an N-glucoside of cis-zeatin from potato tuber sprouts. *Plant Physiology* **109,** 513–516.

Novák O, Hauserová E, Amakorová P, Doležal K, Strnad M. 2008. Cytokinin profiling in plant tissues using ultra-performance liquid chromatography–electrospray tandem mass spectrometry. *Phytochemistry* **69,** 2214–2224.

Novák O, Tarkowski P, Tarkowská D, Doležal K, Lenobel R, Strnad M. 2003. Quantitative analysis of cytokinins in plants by liquid chromatography–single-quadrupole mass spectrometry. *Analytica Chimica Acta* **480,** 207–218.

Ördög V, Stirk WA, Van Staden J, Novák O, Strnad M. 2004. Endogenous cytokinins in three genera of microalgae from the chlorophyta. *Journal of Phycology* **40,** 88–95.

Parker CW, Badenoch-Jones J, Letham DS. 1988. Radioimmunoassay for quantifying the cytokinins cis-zeatin and cis-zeatin riboside and its application to xylem sap samples. *Journal of Plant Growth Regulation* **8,** 93–105.

Persson BC, Bjork GR. 1993. Isolation of the gene (miaE) encoding the hydroxylase involved in the synthesis of 2-methylthio-cis-ribozeatin in tRNA of Salmonella typhimurium and characterization of mutants. *Journal of Bacteriology* **175,** 7776–7785.

Pertry I, Václavíková K, Depuydt S, *et al*. 2009. Identification of *Rhodococcus fascians* cytokinins and their modus operandi to reshape the plant. *Proceedings of the National Academy of Sciences, USA* **106,** 929–934.

Petrášek J, Černá A, Schwarzerová K, Elčkner M, Morris DA, Zažímalová E. 2003. Do phytotropins inhibit auxin efflux by impairing vesicle traffic? *Plant Physiology* **131,** 254–263.

Prinsen E, Redig P, VanDongen W, Esmans EI, VanOnckelen HA. 1995. Quantitative-analysis of cytokinins by electrospray tandem mass-spectrometry. *Rapid Communications in Mass Spectrometry* **9,** 948–953.

Quesnelle PE, Emery RJN. 2007. Cis-cytokinins that predominate in Pisum sativum during early embryogenesis will accelerate embryo growth *in vitro*. *Canadian Journal of Botany* **85,** 91–103.

Romanov GA, Kieber JJ, Schmülling T. 2002. A rapid cytokinin response assay in Arabidopsis indicates a role for phospholipase D in cytokinin signalling. *FEBS Letters* **515,** 39–43.

Romanov GA, Lomin SN, Schmülling T. 2006. Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. *Journal of Experimental Botany* **57,** 4051–4058.

Sakakibara H. 2006. Cytokinins: activity, biosynthesis, and translocation. *Annual Review of Plant Biology* **57,** 431–449.

Saleem M, Lamkemeyer T, Schützenmeister A, Madlung J, Sakai H, Piepho HP, Nordheim A, Hochholdinger F. 2010. Specification of cortical parenchyma and stele of maize primary roots by asymmetric levels of auxin, cytokinin, and cytokinin-regulated proteins. *Plant Physiology* **152,** 4–18.

Schmitz RY, Skoog F, Playtis AJ, Leonard NJ. 1972. Cytokinins: synthesis and biological activity of geometric and position isomers of zeatin. *Plant Physiology* **50,** 702–705.

Schwarzerová K, Petrášek J, Panigrahi KCS, Zelenková S, Opatrný Z, Nick P. 2006. Intranuclear accumulation of plant tubulin in response to low temperature. *Protoplasma* **227,** 185–196.

Simpson MG. 2006. *Plant systematics*. Amsterdam, The Netherlands: Elsevier.

Skoog F, Armstrong DJ. 1970. Cytokinins. *Annual Review of Plant Physiology* **21,** 359–384.

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T. 2004. Two cytokinin receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. *Plant and Cell Physiology* **45,** 1299–1305.

Stirk WA, Novák O, Strnad M, van Staden J. 2003. Cytokinins in macroalgae. *Plant Growth Regulation* **41,** 13–24.

Stirk WA, Novák O, Václavíková K, Tarkowski P, Strnad M, van Staden J. 2008. Spatial and temporal changes in endogenous cytokinins in developing pea roots. *Planta* **227,** 1279–1289.

Sýkorová B, Kurešová G, Daskalova S, Trčková M, Hoyerová K, Raimanová I, Motyka V, Trávníčková A, Elliott MC, Kamínek M. 2008. Senescence-induced ectopic expression of the *A. tumefaciens ipt* gene in wheat delays leaf senescence, increases cytokinin content, nitrate influx, and nitrate reductase activity, but does not affect grain yield. *Journal of Experimental Botany* **59,** 377–387.

Tanaka M, Takei K, Kojima M, Sakakibara H, Mori H. 2006. Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. *The Plant Journal* **45,** 1028–1036.

Tarkowská D, Doležal K, Tarkowski P, Åstot C, Holub J, Fuksová K, Schmülling T, Sandberg G, Strnad M. 2003. Identification of new aromatic cytokinins in Arabidopsis thaliana and Populus×canadensis leaves by LC-(+)ESI-MS and capillary liquid chromatography/frit-fast atom bombardment mass spectrometry. *Physiologia Plantarum* **117,** 579–590.

Tay SAB, MacLeod JK, Palni LMS. 1986. On the reported occurrence of cis-zeatin riboside as a free cytokinin in tobacco shoots. *Plant Science* **43,** 131–134.

Thimann KV, Sachs T. 1964. The role of auxins and cytokinins in the release of buds from dominance. *American Journal of Botany* **54,** 136–144.

Van Staden J, Stirk WA, Novák O, Strnad M. 2010. The role of cytokinins in seed germination. *South African Journal of Botany* **76,** 405.

Veach YK, Martin RC, Mok DWS, Malbeck J, Vankova R, Mok MC. 2003. *O*-Glucosylation of cis-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. *Plant Physiology* **131,** 1374–1380.

Von Schwartzenberg K, Núñez MF, Blaschke H, Dobrev PI, Novák O, Motyka V, Strnad M. 2007. Cytokinins in the bryophyte Physcomitrella patens: analyses of activity, distribution, and cytokinin oxidase/dehydrogenase overexpression reveal the role of extracellular cytokinins. *Plant Physiology* **145,** 786–800.

Vyroubalová Š, Václavíková K, Turečková V, Novák O, Šmehilová M, Hluska T, Ohnoutková L, Frébort I, Galuszka P. 2009. Characterization of new maize genes putatively involved in cytokinin metabolism and their expression during osmotic stress in relation to cytokinin levels. *Plant Physiology* **151,** 433–447.

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T. 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* **15,** 2532–2550.

Werner T, Motyka V, Strnad M, Schmülling T. 2001. Regulation of plant growth by cytokinin. *Proceedings of the National Academy of Sciences, USA* **98,** 10487–10492.

Yonekura-Sakakibara K, Kojima M, Yamaya T, Sakakibara H. 2004. Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to cis-zeatin. *Plant Physiology* **134,** 1654–1661.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. 2004. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiology* **136,** 2621–2632 Erratum in: *Plant Physiology* 2004; **136**, 4335.

Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux

Pernisová, M., **Klíma, P.**, Horák, J., Válková, M., Malbeck, J., Souček, P., Reichman, P., Hoyerová, K., Dubová, J., Friml, J., Zažímalová, E., Hejátko, J. (2009)

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The work on this paper was my side project that arose from collaboration with colleagues from Laboratory of Molecular Plant Physiology at the Faculty of Science, Masaryk University in Brno, my former lab. Paper presents novel views on the molecular basis of interaction of cytokinins and auxins during auxin-induced organogenesis. Briefly, auxin serves as a trigger for new organ formation and locally synthesized CK modulate auxin effects via regulation of the auxin efflux machinery. This CK effect can be simulated by chemical inhibition of the polar auxin transport.

As a member of the team, my task was to test the influence of CKs on auxin efflux using BY-2 suspension-grown cells. 5 µM CKs did not directly influence NAA efflux. Instead, 16 hours pretreatment by various CKs reduced auxin efflux and this reduction was probably caused by regulation of expression of *PIN* genes. I also used AVG (aminoethoxy vinyl glycine, inhibitor of ethylene production) for elimination of possible ethylene mediated effect on auxin transport.

Figure S4 of the Supporting information which displays my work is filed after the article.
Article and Supporting information is freely available online at PNAS website.

Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux

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Postembryonic de novo organogenesis represents an important competence evolved in plants that allows their physiological and developmental adaptation to changing environmental conditions. The phytohormones auxin and cytokinin (CK) are important regulators of the developmental fate of pluripotent plant cells. However, the molecular nature of their interaction(s) in control of plant organogenesis is largely unknown. Here, we show that CK modulates auxin-induced organogenesis (AIO) via regulation of the efflux-dependent intercellular auxin distribution. We used the hypocotyl explants-based in vitro system to study the mechanism underlying de novo organogenesis. We show that auxin, but not CK, is capable of triggering organogenesis in hypocotyl explants. The AIO is accompanied by endogenous CK production and tissue-specific activation of CK signaling. CK affects differential auxin distribution, and the CK-mediated modulation of organogenesis is simulated by inhibition of polar auxin transport. CK reduces auxin efflux from cultured tobacco cells and regulates expression of auxin efflux carriers from the PIN family in hypocotyl explants. Moreover, endogenous CK levels influence *PIN* transcription and are necessary to maintain intercellular auxin distribution *in planta*. Based on these findings, we propose a model in which auxin acts as a trigger of the organogenic processes, whose output is modulated by the endogenously produced CKs. We propose that an important mechanism of this CK action is its effect on auxin distribution via regulation of expression of auxin efflux carriers.

PIN expression | two-component signalling | root meristem | auxin maxima

Postembryonic de novo organogenesis represents an important developmental adaptation evolved in plants. Regeneration of entire bodies in hydras (1) or organs in amphibians (2) has been described. However, in the animal kingdom, these examples are rather exceptional. In contrast, plants evolved postembryonic formation of new organs from differentiated tissues as a strategy that allows physiological and developmental adaptation to changing environmental conditions. However, this strategy requires action by factors that are specifically able to induce developmental programs, leading to the formation of entire organs from virtually differentiated cells.

The interaction of auxin and cytokinin (CK) during plant organogenesis is a phenomenon known for a long time. In their pioneering work, Skoog and Miller (3) identified auxin-to-CK concentration ratios as an important factor regulating the developmental fate of plant tissue explants. Since that time, the role of both growth factors in plant development has been extensively studied. For auxin action, a model involving a spatial and temporal pattern of intercellular auxin distribution and concentration maxima is well established, and the molecular and cellular factors mediating auxin distribution have been identified (4, 5). Differential auxin distribution has been shown to mediate multiple aspects of plant development, such as apical/basal axis formation (6), root patterning (7, 8), tropisms (9–11), and organogenesis (12–15). CK is an important regulator of shoot (16) and root architecture (17–22), and it also regulates seed development (23), abiotic stress (24), and plant senescence (25). CK signaling is mediated by two-component phosphorelay in *Arabidopsis* (for an in-depth recent review, see ref. 26). However, the molecular factors acting downstream of the CK signaling pathway remain mostly unknown.

Here, we use de novo auxin-induced organogenesis (AIO) as a model for characterization of the interactions between CKs and auxin in regulation of plant development. We show that auxin triggers organogenesis and that CK modulates its output through its effect on auxin distribution, which is realized by CK-dependent regulation of expression of auxin transport components.

Results

CK Modulates Auxin-Induced de Novo Organogenesis via Two-Component Signalling. We have used the well-known phenomenon of distinct effects of different CK-to-auxin ratios on the development of plant explants in vitro (3, 27) and adapted this system to study the mechanism underlying de novo organogenesis. Placement of *Arabidopsis* hypocotyls on the media with threshold auxin concentration has resulted in the formation of newly induced root-like organs, even in the absence of exogenous CK. The threshold auxin concentration was identified as the lowest auxin concentration leading to the formation of well-distinguishable organs at different CK concentrations and was identified to be 30 ng/mL (135 nM) for 2,4-dichlorophenoxyacetic acid [2,4-D] and 100 ng/mL (537 nM) for naphthalene-1-acetic acid [NAA] (Fig. 1*A*). In the root-like structures induced by NAA, all important morphological traits of genuine roots could be recognized (i.e., columella, lateral root cap, quiescent center, epidermis, cortex, endodermis, stele). In 2,4-D–induced organs, only the columella-like cells could be distinguished. However, in both 2,4-D– and NAA-induced organs, the columella-like cells revealed DR5 activity, which is consistent with the situation in genuine roots. With an increasing concentration of CK in the media, we observed a decreasing ability of hypocotyl explants to form root-like structures and their gradual disorganization (Fig. 1*A* and Fig. S1, for more details see later in the text). At the CK (kinetin) concentration of 300 ng/mL (1.4 μM, further referred to as the CK threshold), only disorganized callus was produced (Fig. 1*A*), with very rare remnants of distinguishable root-like organs (Fig. S1). After a prolonged period of cultivation at these CK and auxin concentrations, the calli turned green, and new shoots have occasionally been formed from the disorganized tissue (data not shown). However, at auxin concentrations below the organ-inducing threshold, CK alone was unable to induce any organogenic response (Fig. 1 *A* and *B*). This suggests that auxin triggers organogenesis, whereas CK modulates it.

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The authors declare no conflict of interest.

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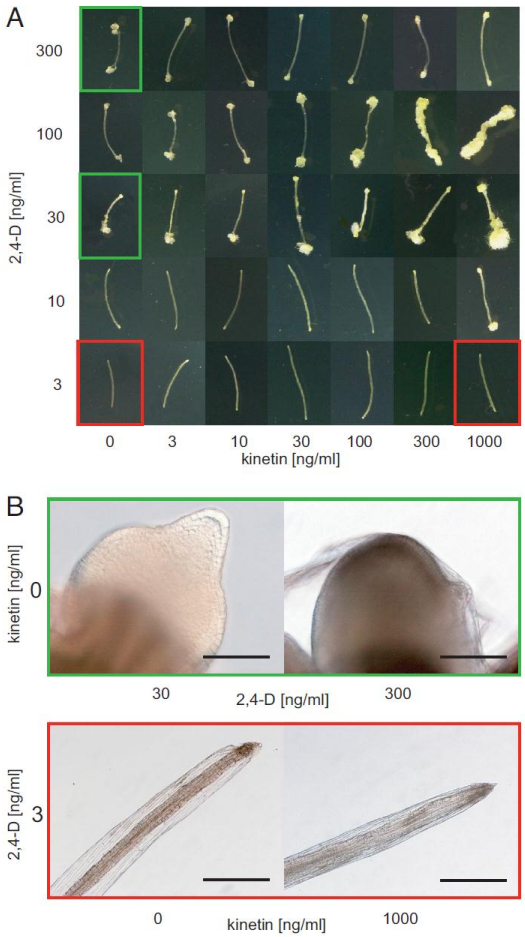

Fig. 1. CK modulates AIO. (*A*) Formation of root-like organs and calli in hypocotyls grown on different combinations of 2,4-D and kinetin. (*B*) Details of the root-like organs (green frames in *A*) formed in the absence of kinetin and at different 2,4-D concentrations. In contrast, no organogenic response was observed at the low auxin concentration and any of the tested kinetin concentrations (red frames in *A*). (Scale bars: 100 μm in green frame and 400 μm in red frame.)

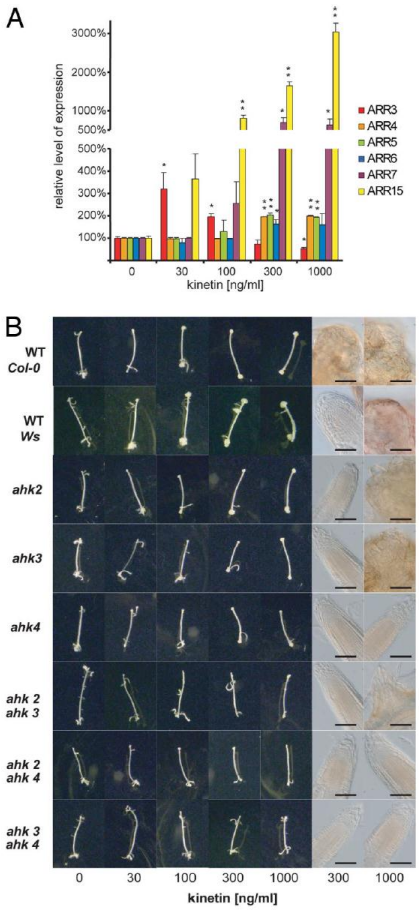

Fig. 2. CK modulates organogenesis via a two-component system. (*A*) Relative expression of CK primary response genes, A-type *ARRs* with the increasing CK concentration in the presence of NAA (537 nM). The statistical significance of identified differences in comparison to the absence of exogenous CKs (*t* test) at alpha 0.05 and 0.01 is designated (* and **, respectively); error bars show SDs. (*B*) Phenotypes of root-like organs induced by NAA (537 nM) at the increasing CK concentrations in WT (*Col-0* and *Ws* ecotypes) and different single and double CK receptor mutants. All mutants are of *Col-0* ecotype except for *ahk4* and *ahk3 ahk4*, which carry *ahk4–1* allele from *Ws* (see *Materials and Methods*). (Scale bar: 50 μm.)

To address the involvement of CK signaling in the observed phenomenon, we analyzed expression of the primary CK response genes (i.e., the A-type *ARR* genes) (28) in hypocotyl explants using quantitative real-time (qRT) PCR in the presence of auxin threshold [NAA (100 ng/mL)] and increasing CK concentration. With the exception of *ARR3*, which peaked at 30 ng/mL (139 nM) kinetin, we have found a gradual increase in the expression levels of all inspected A-type *ARRs* with increasing CK concentration; a particularly steep increase of expression was observed at the CK threshold concentration (Fig. 2*A*). Next, we addressed the involvement of CK perception and its specificity in the observed morphogenic effect. We analyzed the organogenic response in hypocotyl explants isolated from mutants in CK receptors AHK2, AHK3, and AHK4 (29, 30). All single, and particularly double, mutants showed increased resistance to CK in terms of modulation of organogenesis in comparison to corresponding WT (Fig. 2*B*). Differences in the strength of the phenotype in particular single and double *ahk* mutants suggest a certain specificity of individual signaling pathways in the CK-dependent modulation of organogenesis, with a dominant effect of *AHK4*, followed by *AHK3* and *AHK2* (*AHK4* ≥ *AHK3* > *AHK2*; Fig. 2*B*). These findings are in accordance with previous observations (29, 30), thus confirming the suitability of our experimental setup. Collectively, these results show that CKs modulate the auxin-induced organogenic response in *Arabidopsis* via two-component signaling.

AIO Is Accompanied by the Production of Endogenous CKs and Tissue-Specific Activation of CK Signaling. The expression of A-type *ARR*s even in the absence of exogenous CKs (Fig. 2*A* and data not shown) suggests that the AIO might be accompanied by endogenous CK production and subsequent activation of CK signaling. To identify the potential importance of endogenous CKs in AIO, we

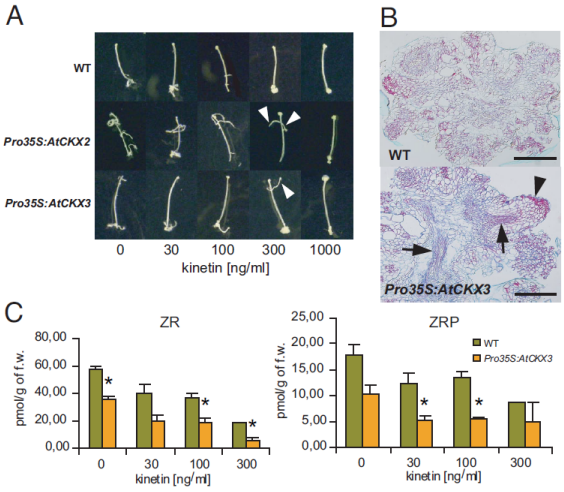

Fig. 3. Auxin induces production of endogenous CKs that contribute to AIO. (*A*) Formation of root-like organs induced by NAA (537 nM). Note that in *Pro35S:AtCKX2* and *Pro35S:AtCKX3* lines, there are still root-like organs distinguishable even at the CK threshold concentration (arrowheads), which is not the case in WT. (*B*) Structure of calli induced by auxin (537 nM NAA) at the CK threshold (1.4 μM kinetin). In the *Pro35S:AtCKX3* line, there are still patterned organs distinguishable (arrowhead) in comparison to WT, where only almost completely disorganized tissue could be detected. Arrows point to the patterned vascular tissue in *Pro35S:AtCKX3* calli. (Scale bar: 200 μm.) (*C*) Levels of endogenous CKs after induction of organogenesis by NAA (537 nM) at different exogenous CK concentrations. The statistical significance of identified differences in comparison to WT (*t* test) at alpha 0.05 is designated (*); error bars show SDs. For the data on all analyzed CK metabolites, see Fig. S2 and Table S1.

inspected organogenesis in hypocotyl explants with endogenous CKs depleted via ectopic overexpression of *CYTOKININ OXIDASE/DEHYDROGENASE* genes (19). In *Pro35S:AtCKX2* and *Pro35S:AtCKX3* explants, we observed partial resistance to CK, as manifested by increased competence of hypocotyl explants to form root-like organs (Fig. S1) and formation of root-like structures even at the CK threshold concentration (Fig. 3*A* and *B*). Because kinetin has been found to be only a poor substrate of CKX (31), this effect seems to be attributable to a decrease of endogenous CKs rather than to inactivation of exogenously applied CKs. To confirm that, we have measured levels of endogenous CKs in hypocotyl explants cultivated in the absence and presence of exogenous CKs. In the WT hypocotyl explants, endogenous CKs [from active CKs, predominantly *trans*-zeatin-9-riboside (ZR) and ZR phosphate] were found in the hypocotyls grown at the organogenesis-inducing (threshold) auxin concentration in the absence of exogenous CK. The amounts of most of the endogenous CKs were substantially reduced in the *Pro35S:AtCKX3* hypocotyl explants (Fig. 3*C* and Fig. S2). Surprisingly, the addition of exogenous CKs led to the further reduction of endogenously produced CKs in both WT and *AtCKX3* overexpressing hypocotyl explants (Fig. 3*C*). This is presumably attributable to up-regulation of endogenous *AtCKX* expression by exogenous CKs (32). These data show that AIO is accompanied by the production of endogenous CKs that affect its developmental output.

To gain insight into the potential tissue specificity of CK production and action during AIO, we have inspected expression of *ARR5*, one of the earliest expressed CK primary response genes (28). In agreement with our qRT-PCR data, we have observed the activity of *ARR5* promoter in *ProARR5:GUS* hypocotyl explants even in the absence of exogenous CKs (Fig. S3*A*). GUS activity in hypocotyl explants was delimited to the induced root-like organs, suggesting tissue specificity of CK signaling leading to up-regulation of *ARR5* expression. Expression of *ARR5* was reduced in both *Pro35S:AtCKX2* and *Pro35S:AtCKX3* lines (Fig. S3*A* and data not shown, respectively). Altogether, these findings indicate that AIO is accompanied by tissue-specific activation of the CK signaling pathway and endogenous CK production that contributes to the CK-dependent modulation of AIO. Thus, the CK effect on AIO in our system is a sum of both endogenous and exogenous CKs.

CK Affects Auxin Distribution During de Novo Organogenesis. To identify a mechanism of CK action during AIO, we inspected its effect on the morphology of auxin-induced organs in more detail. Interestingly, we found important differences in CK effect on AIO induced by either 2,4-D or NAA. In the absence of exogenous CKs, NAA induces formation of root-like structures with a cellular pattern that resembles *Arabidopsis* roots. The increasing CK concentration led to a decrease in the number of NAA-induced organs (Fig. S1); however, the morphology of formed organs was only slightly affected by CK concentration below the CK threshold (Fig. 4*A*). In contrast, 2,4-D induced formation of only poorly specified root-like organs that only partially resembled *Arabidopsis* roots, and the increasing CK concentration led to a gradual loss of organ structure and patterning (Fig. 4*A*). However, in both cases, the CK threshold led to the loss of organ formation and only unorganized callus was formed (Fig. 4*A*). The 2 types of auxin used, 2,4-D and NAA, differ in the mechanism of their transport in plant cells. While 2,4-D must be taken up into cells actively by AUX/LAX importers (11), NAA enters cells almost entirely via passive diffusion (33). On the other hand, NAA, but not 2,4-D, gets out from cells easily via auxin efflux carriers (33, 34), and can thus be more efficiently transported between cells. Thus, these different effects of CK on NAA- and 2,4-D–induced organogenesis indicated the involvement of auxin transport in this process.

Transport-dependent control of the spatial and temporal pattern of auxin distribution in plant tissues plays an important role in multiple aspects of organogenesis *in planta* (13). Thus, we examined the potential CK effect on the formation of local auxin maxima as visualized by the activity of the auxin response reporter DR5 (35) in organs induced by 2,4-D or NAA. The NAA-induced organs displayed single auxin maxima at the "root tip," which resembles the situation in *Arabidopsis* root primordia (13, 36). With an increasing CK concentration, the auxin maxima in NAA-induced organs were only slightly affected; they became diffuse and weaker, as visible particularly in *DR5rev:GUS* (Fig. 4*B*). On the other hand, 2,4-D–induced organs formed with multiple ectopically located auxin maxima in additional "root tips". The increasing CK concentration resulted in the formation of less focused auxin maxima and their spreading and disorganization. That correlated well with changes in the shape of 2,4-D–induced root-like organs, (i.e., gradual loss of the organ structure and patterning) (Fig. 4*B*). At the CK-threshold concentration, almost complete loss of auxin maxima formation was observed in both NAA- and 2,4-D–induced calli (Fig. 4*B*). Thus, the apparently higher sensitivity of 2,4-D–induced organs to CK-mediated morphogenic effect very probably reflects lower efficiency of efflux carriers to relocate 2,4-D in comparison to NAA. However, at concentrations reaching or higher than the CK threshold, the auxin efflux capacity decreases below the level necessary for formation of defined auxin maxima and, consequently, results in loss of organ patterning in both 2,4-D– and NAA-induced organogenesis.

Moreover, treatment with 1-naphthylphthalamic acid (NPA), a potent inhibitor of polar auxin transport at the level of auxin efflux (37, 38), partially mimics the effect of exogenous CKs (Fig. 4*C*). In the presence of NPA (10 μM) and absence of exogenous CKs, NAA induces the formation of root-like organs similar to those induced by 2,4-D. However, these organs were more sensitive to both endogenous and exogenous CKs. That was manifested by the formation of a large amount of callus and a higher degree of organ

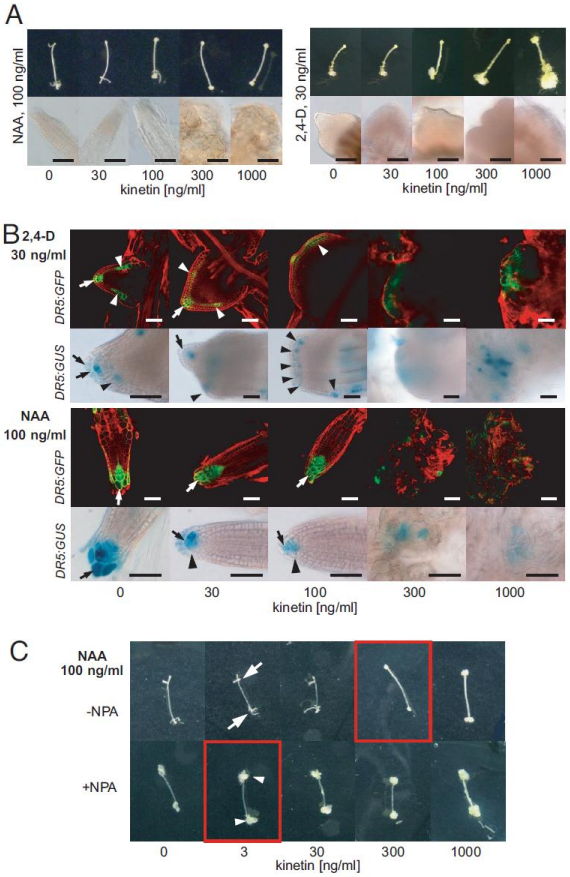

Fig. 4. CK has effects on auxin distribution during de novo organogenesis. (*A*) Phenotype of root-like organs after induction of organogenesis in hypocotyl explants either by NAA (*Left*) or 2,4-D (*Right*). Note the higher organization and patterning resembling *Arabidopsis* roots in the case of root-like organs induced by NAA. (Scale bar: 100 μm.) (*B*) Spatial pattern of auxin maxima as visualized by activity of DR5 on 2,4-D– (*Upper*) and NAA-induced organs (*Lower*). Note the formation of ectopic auxin maxima (arrowheads), their spreading and disorganization, and the more pronounced loss of the organ patterning and structure in the case of organs induced by 2,4-D. The auxin maxima are gradually less pronounced in case of NAA-induced root-like organs (arrowhead). Note the almost complete disappearance of DR5 maxima and loss of organ patterning at the CK threshold concentration [300 ng/mL (1.4 μM) kinetin]. Arrows depict the columella-like cells. (Scale bar: 50 μm.) (*C*) NPA (10 μM) partially mimics the effect of the CK threshold. Note the large amount of calli (arrowheads) formed even at the lowest kinetin concentration [3 ng/mL (14 nM)] in comparison to well-recognizable root-like organs at the same kinetin concentration in the absence of NPA (arrows); this resembles the effect of the CK threshold in the absence of NPA (compare figures in red frames). For details, see Fig. S3.

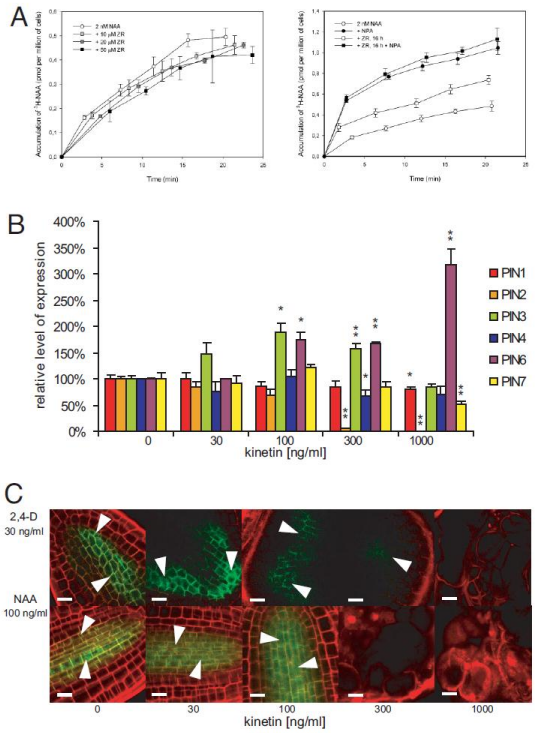

Fig. 5. CKs modulate auxin efflux and *PIN* expression. (*A*) Accumulation of [^{3}H]NAA in BY-2 cells. Although there is no apparent effect on the auxin accumulation in BY-2 cells when CK of different concentrations (10, 20, and 50 μM ZR) was added together with [^{3}H]NAA (*Left*), the 16-hour pretreatment with 5 μM ZR led to an increase in the accumulation of [^{3}H]NAA (*Right*); 2 nM [^{3}H]NAA was used in both cases. This effect could be mimicked by addition of 10 μM NPA (*Right*). Error bars show SDs. (*B*) Relative transcription of *PIN* auxin transporters in hypocotyl explants at different CK concentrations and at the auxin threshold (NAA; 537 nM) as measured by qRT-PCR. The statistical significance of identified differences in comparison to the absence of exogenous CKs (*t* test) at alpha 0.05 and 0.01 is designated (* and **, respectively), and error bars show SDs. (*C*) PIN1-GFP signal (green, arrowheads) in the root-like organs of *ProPIN1:PIN1-GFP* hypocotyl explants at the different CK concentrations. Note that the signal is getting weaker and diffuse and disappears at the CK threshold in the case of both 2,4-D and NAA. (Scale bar: 10 μm.)

structure disintegration even at the lowest CK concentration applied, thus resembling the CK threshold (Fig. 4*C* and Fig. S3). Accordingly, in the absence of exogenous CKs, NPA led to the formation of organs whose structure was better preserved and more resembled roots in *Pro35S:AtCKX2* hypocotyl explants in comparison to WT (Fig. S3*B*). This suggests partially synergistic but distinct effects of NPA and CKs on the AIO. Taken together, our results indicate a correlation between auxin distribution and its organogenic effect during de novo AIO and suggest an interference of CKs with the formation of the cellular efflux-dependent local auxin maxima. Nevertheless, it is obvious, and must be considered, that apart from the role of CKs in the regulation of auxin distribution, CKs also affect organogenesis via other mechanisms (e.g., regulation of cell proliferation) (3).

CK Affects the Expression of *PIN* Auxin Efflux Carriers and the Cellular Efflux of Auxin. Our results suggest that, as shown for *in planta* organogenesis (12, 13, 15), auxin efflux is also required for control of auxin distribution during de novo-induced organogenesis in our in vitro system. Therefore, we tested the scenario that CK influences AIO through an effect on auxin efflux. Accumulation of radioactively labeled NAA ([^{3}H]NAA) in cultured tobacco BY-2 cells is a reliable system for the measuring and quantification of auxin efflux (34). When CKs were added to the cell suspension just at the beginning of the assay (i.e., together with [^{3}H]NAA), no CK effect on the [^{3}H]NAA accumulation was observed. However, pretreatment of BY-2 cells with CKs (5 μM ZR for 16 h) led to the increase of the [^{3}H]NAA accumulation (i.e., to a decrease of its efflux) (Fig. 5*A*). Similar results were obtained with various other CKs (Fig. S4). Thus, CKs similar to established auxin transport inhibitors, such as NPA, inhibit auxin efflux. However, the long lag

time of CK effect suggests that CKs regulate auxin efflux in BY-2 cells via regulation of expression of genes for efflux carriers or regulatory proteins rather than via direct interference with efflux activity. CK enhances ethylene biosynthesis (39), and the involvement of ethylene in the regulation of auxin transport has been reported (40, 41). Therefore, we analyzed CK effects on the auxin efflux in the presence of aminoethoxy vinyl glycine (AVG), an inhibitor of ethylene production (42). No difference in auxin accumulation in BY-2 cells treated with CK was observed between the absence and presence of AVG (Fig. S4), showing that CKs act on auxin efflux independent of regulation of ethylene biosynthesis.

Next, we addressed the possible mechanisms by which CKs modulate auxin efflux. Auxin carriers from the PIN family were identified to be the rate-limiting regulators of the cellular auxin efflux (34), and their key role in generating differential local auxin distribution has been demonstrated (5). Because cellular output of CK signaling occurs at the level of regulation of gene expression, we tested possible regulation of *PIN* expression by CKs. Using qRT-PCR, we have observed differential transcription of individual *PIN* genes in hypocotyl explants cultivated in the presence of auxin threshold (537 nM NAA) and different CK concentrations. Although the expression of *PIN3* peaked at 100 ng/mL CK (464 nM kinetin) and decreased with further increasing CK concentrations, the expression of *PIN6* was up-regulated at the same CK concentration (100 ng/mL) and further increased at 1,000 ng/mL CK (4.6 μM kinetin) (Fig. 5*B*). The transcription of root-specific *PIN2* (10) dramatically decreased at the CK threshold, presumably reflecting the loss of root identity and formation of only undifferentiated calli (Fig. 5*B*). Interestingly, *PIN1* transcription was only slightly down-regulated even at the highest CK concentration (4.6 μM kinetin) (Fig. 5*B*). However, in both 2,4-D– and NAA-induced root-like organs, the signal of PIN1-GFP was getting weaker and more diffuse with the increasing CK concentrations (Fig. 5*C*). The PIN1-GFP signal was lost in calli at the CK threshold concentration, and only residual PIN1-GFP, apparently not associated with plasma membrane, was occasionally detectable (data not shown); for quantification of the CK effect on *PIN1-GFP* expression, see Fig. S5. Thus, CKs seem to affect the expression of *PIN* genes, possibly at both transcriptional and posttranscriptional levels. Taken together, these results show that CKs regulate expression of PIN auxin efflux carriers during de novo AIO, which provides a plausible mechanism for CK-dependent regulation of auxin efflux.

Endogenous CKs Are Required for Differential Auxin Distribution in *Arabidopsis* Roots. Our results imply that CKs can affect auxin distribution during de novo organogenesis via regulation of auxin efflux from cells. In root development, differential auxin distribution has been shown to regulate activity and patterning of the root meristem (7, 36). Thus, we addressed whether endogenous CKs are required for auxin distribution and root meristem patterning *in planta*. We examined the formation of local auxin maxima (visualized by DR5 activity) in CK-deficient *Pro35S:AtCKX2* and *Pro35S:AtCKX3* plants. In the root tips of these plants, *DR5rev:GFP* expression in columella expanded more laterally in comparison to that of control (Fig. 6*A*). We analyzed 2 lines of each transformant, *Pro35S::AtCKX2* and *Pro35S::AtCKX3*. For *Pro35S::AtCKX2*, 30 and 33 aberrant roots were scored out of 38 and 40 inspected roots, respectively (30 of 38 roots and 33 of 40 roots). For *Pro35S::AtCKX3*, the result was similar (32 of 41 roots and 19 of 23 roots). In WT background, only 5 of 39 inspected roots revealed aberrations in the *DR5rev:GFP* expression pattern. Accordingly, the first 5 columella cells were significantly enlarged in the longitudinal direction in several independent *Pro35S:AtCKX2* and *Pro35S:AtCKX3* lines (Fig. S6). This presumably reflects the dose-dependent role of auxin in the regulation of cell elongation (43) and provides additional evidence for a disturbed auxin gradient in the root tip of *Pro35S:AtCKX2(3)* lines. We also tested whether endogenous CK levels influence *PIN* transcription or polar PIN

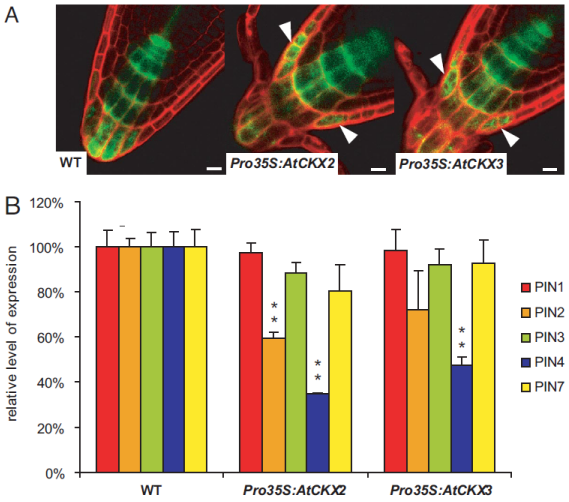

Fig. 6. Endogenous CK levels are required for local auxin maxima formation and mediate *PIN* gene expression in *Arabidopsis* roots. (*A*) Depletion of endogenous CKs in *Pro35S:AtCKX2* and *Pro35S:AtCKX3* lines leads to the defects in auxin response gradients as visualized by *DR5rev:GFP*. Note the lateral expansion of the maxima in transgenic lines (arrowheads) in comparison to WT. (Scale bar: 10 μm.) (*B*) Relative *PIN* transcription measured by qRT-PCR in *Pro35S:AtCKX2* and *Pro35S:AtCKX3* roots in comparison to WT. The statistical significance of differences (*t* test) at alpha 0.01 is marked by **; error bars show SDs.

localization. In the roots of 6-day-old seedlings of both *Pro35S:AtCKX2* and *Pro35S:AtCKX3* lines, polar localization of PIN2 and PIN4 proteins did not differ from that of controls (Fig. S7), but we have found a strong decrease in the *PIN2* and *PIN4* mRNA levels (Fig. 6*B*). Based on these data, we conclude that distinct levels of endogenous CKs are necessary to maintain expression of *PIN* auxin efflux carriers in the root tip, thus regulating formation of local auxin maxima and root meristem development. These data show that the mechanism of CK-dependent regulation of *PIN* transcription and control of differential auxin distribution that we identified during de novo organogenesis also applies for processes *in planta*.

Discussion

Our work addresses the mechanism underlying the role of the phytohormones auxin and CK in plant organogenesis. We show that in contrast to CK, auxin is able to induce a de novo organogenic response in hypocotyl explants. This is in accordance with the recent recognition of auxin and/or its gradients as a general trigger for the change in the developmental program in plants (4, 15). We have found that the auxin-induced organogenic response is accompanied by production of endogenous CKs and the tissue-specific activation of the CK signaling pathway. The activation of *ARR5* expression in the absence of exogenous CKs was also observed in root explants (44). This further confirms our conclusions and implies that auxin might induce similar developmental programs in root and hypocotyl explants, thus strengthening the role of auxin as a universal trigger of organogenesis.

Formation of lateral roots represents one of the examples for postembryonal de novo organogenesis in plants. Recent reports (20, 22) suggest potential involvement of CKs in the regulation of auxin efflux during lateral root formation. Exogenous CKs are supposed to down-regulate expression of all inspected *PIN* genes at early stages of lateral root primordia development (20). However, this does not seem to be the case in the roots of *Pro35S:AtCKX2(3)* lines,

in which at least *PIN2* and *PIN4* are down-regulated after endogenous CK depletion. Our results reflect predominantly the context of primary root meristem, because we have analyzed *PIN* expression in the roots of 6-day-old seedlings, in which only a few lateral roots and lateral root primordia have yet been formed. This implies that CKs affect the expression of individual PIN carriers differentially in particular plant tissues and that complex interactions between CKs and individual members of the auxin-efflux machinery should be further characterized in a spatiotemporal context.

Our results suggest that in addition to recently identified interaction between CK and auxin on the level of signaling (45), CKs modulate auxin distribution via regulation of auxin efflux. This type of regulation represents a thus far unidentified mechanism for well-known CK-auxin interactions during plant development. We propose that changes in endogenous CK levels form an intrinsic part of the auxin-induced organogenic response and that CK-mediated modulation of auxin distribution via regulation of auxin efflux is one of the mechanisms underlying the auxin-CK interaction during organogenesis in plants.

Materials and Methods

Plant Materials. Unless otherwise stated, all plant material used was *Arabidopsis thaliana*, ecotype *Col-0*. For the hypocotyl explant assay, *ahk2–1*, *ahk3–1*, and *ahk4–1* (30) single-mutant lines and *ahk2–1 ahk3–1*, *ahk3–1 ahk4–1* (30), and *ahk2–2TK cre1–12* (29) double-mutant lines were used. For details of preparation of transgenic lines used, see *SI Text*.

Hypocotyl Explants Assay. Plants were cultivated 1 day in the light and 5 days in the dark in Petri dishes with Murashige and Skoog medium, including Gamborg B5 vitamins in growth chambers (Percival) at 21 °C. Hypocotyls were isolated by removing cotyledons and roots and were placed on Petri dishes with cultivation medium as described (27) and enriched with respective hormone concentrations. Kinetin, 2,4-D, and NAA were purchased from Sigma-Aldrich. Hypocotyl explants were cultivated for 21 days under long-day conditions (16 h light at 21 °C and 8 h dark at 19 °C), a light intensity of 100 μM·m^{-2}·s^{-1}, and 80% relative humidity.

[^{3}H]NAA Accumulation in BY-2 Cells. The [3H]NAA accumulation assay was performed as described (38).

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1. Gierer A, *et al.* (1972) Regeneration of hydra from reaggregated cells. *Nat New Biol* 239:98–101.
2. Endo T, Bryant SV, Gardiner DM (2004) A stepwise model system for limb regeneration. *Dev Biol* 270:135–145.
3. Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp Soc Exp Biol* 54:118–130.
4. Vieten A, Sauer M, Brewer PB, Friml J (2007) Molecular and cellular aspects of auxin-transport-mediated development. *Trends Plant Sci* 12:160–168.
5. Tanaka H, Dhonukshe P, Brewer PB, Friml J (2006) Spatiotemporal asymmetric auxin distribution: A means to coordinate plant development. *Cell Mol Life Sci* 63:2738–2754.
6. Friml J, *et al.* (2003) Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. *Nature* 426:147–153.
7. Friml J, *et al.* (2002) AtPIN4 mediates sink-driven auxin gradients and root patterning in Arabidopsis. *Cell* 108:661–673.
8. Blilou I, *et al.* (2005) The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature* 433:39–44.
9. Friml J, Wisniewska J, Benkova E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in Arabidopsis. *Nature* 415:806–809.
10. Luschnig C, Gaxiola RA, Grisafi P, Fink GR (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in Arabidopsis thaliana. *Genes Dev* 12:2175–2187.
11. Marchant A, *et al.* (1999) AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. *EMBO J* 18:2066–2073.
12. Heisler MG, *et al.* (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. *Curr Biol* 15:1899–1911.
13. Benkova E, *et al.* (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* 115:591–602.
14. Reinhardt D, *et al.* (2003) Regulation of phyllotaxis by polar auxin transport. *Nature* 426:255–260.
15. Dubrovsky JG, *et al.* (2008) Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. *Proc Natl Acad Sci USA* 105:8790–8794.
16. Kurakawa T, *et al.* (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445:652–655.
17. Scheres B, *et al.* (1995) Mutations affecting the radial organization of the Arabidopsis root display specific defects throughout the embryonic axis. *Development* 121(1):53–62.
18. Mahonen AP, *et al.* (2006) Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. *Science* 311:94–98.
19. Werner T, *et al.* (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* 15:2532–2550.
20. Laplaze L, *et al.* (2007) Cytokinins act directly on lateral root founder cells to inhibit root initiation. *Plant Cell* 19:3889–3900.
21. Dello Ioio R, *et al.* (2007) Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. *Curr Biol* 17:678–682.
22. Kuderova A, *et al.* (2008) Effects of conditional IPT-dependent cytokinin overproduction on root architecture of Arabidopsis seedlings. *Plant Cell Physiol* 49:570–582.
23. Riefler M, Novak O, Strnad M, Schmulling T (2006) Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18:40–54.
24. Tran LS, *et al.* (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. *Proc Natl Acad Sci USA* 104:20623–20628.
25. Kim HJ, *et al.* (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. *Proc Natl Acad Sci USA* 103:814–819.
26. To JP, Kieber JJ (2008) Cytokinin signaling: Two-components and more. *Trends Plant Sci* 13:85–92.
27. Kubo M, Kakimoto T (2000) The cytokinin-hypersensitive genes of Arabidopsis negatively regulate the cytokinin-signaling pathway for cell division and chloroplast development. *Plant J* 23:385–394.
28. D'Agostino IB, Deruere J, Kieber JJ (2000) Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiol* 124:1706–1717.
29. Higuchi M, *et al.* (2004) In planta functions of the Arabidopsis cytokinin receptor family. *Proc Natl Acad Sci USA* 101:8821–8826.
30. Nishimura C, *et al.* (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. *Plant Cell* 16:1365–1377.
31. Popelkova H, *et al.* (2006) Kinetic and chemical analyses of the cytokinin dehydrogenase-catalysed reaction: Correlations with the crystal structure. *Biochem J* 398:113–124.
32. Werner T, Kollmer I, Bartrina I, Holst K, Schmulling T (2006) New insights into the biology of cytokinin degradation. *Plant Biology* 8:371–381.
33. Delbarre A, Muller P, Imhoff V, Guern J (1996) Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. *Planta* 198:532–541.
34. Petrasek J, *et al.* (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* 312:914–918.
35. Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* 9:1963–1971.
36. Sabatini S, *et al.* (1999) An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. *Cell* 99:463–472.
37. Katekar GF, Geissler AE (1980) Auxin transport inhibitors: IV. Evidence of a common mode of action for a proposed class of auxin transport inhibitors: The phytotropins. *Plant Physiol* 66:1190–1195.
38. Petrasek J, *et al.* (2003) Do phytotropins inhibit auxin efflux by impairing vesicle traffic? *Plant Physiol* 131:254–263.
39. Chae HS, Faure F, Kieber JJ (2003) The eto1, eto2, and eto3 mutations and cytokinin treatment increase ethylene biosynthesis in Arabidopsis by increasing the stability of ACS protein. *Plant Cell* 15:545–559.
40. Ruzicka K, *et al.* (2007) Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. *Plant Cell* 19:2197–2212.
41. Swarup R, *et al.* (2007) Ethylene upregulates auxin biosynthesis in Arabidopsis seedlings to enhance inhibition of root cell elongation. *Plant Cell* 19:2186–2196.
42. Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher-plants. *Annu Rev Plant Physiol Plant Mol Biol* 35:155–189.
43. Hardtke CS (2007) Transcriptional auxin-brassinosteroid crosstalk: Who's talking? *BioEssays* 29:1115–1123.
44. Gordon SP, *et al.* (2007) Pattern formation during de novo assembly of the Arabidopsis shoot meristem. *Development* 134:3539–3548.
45. Muller B, Sheen J (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453:1094–1097.

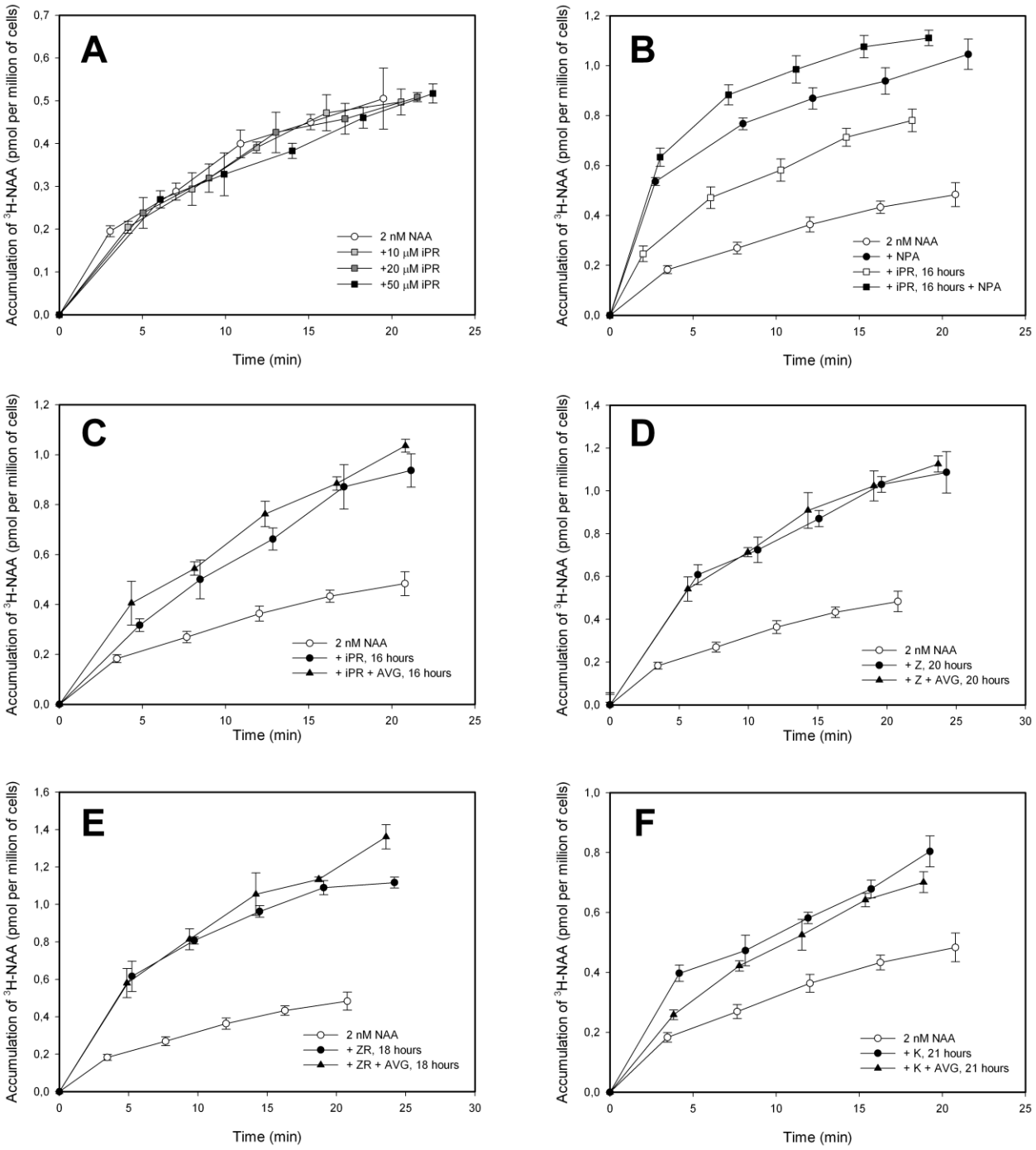

Fig. S4. CKs modulate auxin efflux in BY-2 cells independent of ethylene production. Effect of CK on [^{3}H]NAA accumulation in BY-2 cells. (*A*) Effect of immediate addition of CK at different concentrations. (*B–F*) Effect of pretreatment with different CKs (5 μM) in the presence and absence of the ethylene biosynthesis inhibitor AVG (10 μM). Note that there is no difference in the absence and presence of AVG, suggesting an ethylene-independent effect of CK on auxin efflux in BY-2 cells. iPR, isopentenyl adenosine; Z, *trans*-zeatin; ZR, *trans*-zeatin riboside; K, kinetin. Error bars show SDs.

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  - Symposium on Plant cell elongation: integration of hormonal and environmental signals, 20th December 2007 Ghent, Belgium
  - Plant Transformation Technologies, 4th –7th February 2007, Vienna, Austria
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- Member of the International Plant Growth Substances Association.

Certified languages

English, French

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