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ISOLATION, IDENTIFICATION AND QUANTIFICATION OF CYTOKININ NUCLEOTIDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS

Summary of the Ph.D. thesis
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Content

Declaration I

Hereby I declare that the Ph.D. thesis is my original work. The literature used is listed in the References section.

In Olomouc, 11.02.2011

Tibor Béres

List of papers

The thesis is based on the following papers. These are referred in the text by the corresponding roman numerals and are enclosed in the Supplement section:

I. Voller J., Zatloukal M., Lenobel R., Dolezal K., **Beres T.**, Krystof V., Spichal L., Niemann P., Dzubak P., Hajduch M., Strnad M. Anticancer activity of natural cytokinins: a structure-activity relationship study. *Phytochemistry* **71** 1350-1359 (2010).

II. **Beres T.**, Zatloukal M., Voller J., Niemann P., Christin Gahsche M., Tarkowski P., Novak O., Hanus J., Strnad M., Dolezal K. Tandem mass spectrometry identification and LC-MS quantification of intact cytokinin nucleotides in K-562 human leukemia cells. *Anal. Bioanal. Chem.,* **398** 2071-2080 (2010).

III. Kowalska M., Galuszka P., Frebortova J., Sebela M., **Beres T.**, Hluska T., Smehilova M., Bilyeu K.D., Frebort I. Vacuolar and cytosolic cytokinin dehydrogenases of Arabidopsis thaliana: Heterologous expression, purification and properties. *Phytochemistry* **71** 1970-1978 (2010).

IV. **Beres T.*** & Gemrotova M.*, Tarkowski P., Ganzera M., Maier V., Spichal L., Friedecky D., Strnad M., Dolezal K. Assaying the enzyme activity of recombinant Arabidopsis thaliana isopentenyltransferase 1 (AtIPT1) by capillary electrophoresis (manuscript in preparation).
*Equal contribution

Declaration II

I declare that my role in preparation of the papers listed above, was as following:

I. Co-author – capillary electrophoresis measurements

II. First author – HPLC-MS and HPLC-MS/MS method development, optimalization and validation, manuscript preparation

III. Co-author – capillary electrophoresis measurements

IV. Joined first author – capillary electrophoresis method development, optimalization and validation, HPLC-MS measurements, manuscript preparation

Abbreviations

ABA	abscisic acid
ACN	acetonitrile
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
APCI	atmospheric pressure chemical ionization
*At*CKX	*Arabidopsis thaliana* cytokinin dehydrogenase
*At*IPT	*Arabidopsis thaliana* isopentenyltransferase
ATP	adenosine-5'-triphosphate
BAPR	6-benzylaminopurine riboside
BGE	background electrolyte
CE	capillary electrophoresis
CE-LIF	capillary electrophoresis – laser induced fluorescence
CEM	T-lymphoblastic leukemia
CE-MS	capillary electrophoresis – mass spectrometry
CE-UV	capillary electrophoresis – UV
CI	chemical ionization
CK	cytokinin
CKX	cytokinin dehydrogenase
CTAB	cetyltrimethylammonium bromide
CYP735A	cytochrome P450 monooxygenase
cZ	*cis*-zeatin
DAD	diode array detector
DHZ	dihydrozeatin
DMAPP	dimethylallyldiphosphate
DZOG	dihydrozeatin-O-glucoside
EI	electron ionization
ELISA	enzyme-linked immunosorbent assay
EOF	electroosmotic flow
ESI	electrospray ionization
FAB	fast atom bombardment
GA_1	gibberellin A1
GA_3	gibberellic acid; gibberellin A3
GABA	γ-aminobutyric acid
GC	gas chromatography
HL-60	human promyelocytic leukemia cells
HMBDP	hydroxymethylbutenyldiphosphate
HPLC	high performance liquid chromatography
HPLC-MS	high performance liquid chromatography – mass spectrometry
HPLC-MS/MS	high performance liquid chromatography – tandem mass spectrometry
HPLC-QqTOF-MS	high performance liquid chromatography – quadrupole time-of-flight mass spectrometry
IAA	indole-3-acetic acid
IAAsp	indole-3-acetylaspartic acid
IAC	immunoaffinity chromatography
IEC	ion exchange chromatography
i.d.	internal diameter
iP	N^6-(Δ^2-isopentenyl)adenine
iPDP	N^6-(Δ^2-isopentenyl)adenosine 5'-diphosphate
iPMP	N^6-(Δ^2-isopentenyl)adenosine 5'-monophosphate
iPR	N^6-(Δ^2-isopentenyl)adenosine
iPTP	N^6-(Δ^2-isopentenyl)adenosine 5'-triphosphate
ipt	Isopentenyltransferase
IS	internal standard
IT	ion trap
K	kinetin

K562	chronic myelogenous leukaemia
KR	kinetin riboside
LC	liquid chromatography
LC-MS	liquid chromatography with mass spectrometric detection
LIF	laser induced fluorescence
LOD	limit of detection
LOG	the “Lonely Guy”
LVSS	large volume sample stacking
MALDI	matrix assisted laser desorption ionization
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MEP	methylerythritolphosphate
MRM	multiple reaction monitoring
MS	mass spectrometry
MS^n	multiple steps of MS analysis
MVA	mevalonate
m/z	mass-to-charge ratio
NP	normal phase
oT	*ortho*-topolin
oTR	*ortho*-topolin riboside
oTRDP	*ortho*-topolin riboside-5’-diphosphate
oTRMP	*ortho*-topolin riboside-5’-monophosphate
oTRTP	*ortho*-topolin riboside-5’-triphosphate
P450	P450 monooxygenase
PC	paper chromatography
PDA	photo diode array
PF	partial filling
Q-TOF	quadrupole time-of-flight
QqQ	triple quadrupole
RIA	radioimmunoassay
RP	reversed phase
SIM	selected ion monitoring
SPE	solid phase extraction
TLC	thin layer chromatography
TOF	time of flight
tRNA	transfer ribonucleic acid
t_R	retention time
TS	thermospray
tZ	*trans*-zeatin
tZMP	*trans*-zeatin riboside-5’-monophosphate
tZOG	*trans*-zeatin-O-glucoside
tZR	*trans*-zeatin riboside
UHPLC	ultra high performance liquid chromatography
UV	ultraviolet

1 Introduction

The regulation of plant growth and development is based on chemical signalling as plants miss a nervous system, typical for animals. Phytohormones are the messengers and the changes in their levels accompany physiological processes. For a plant biologist, it is often essential to follow these variations in phytohormone concentrations to elucidate an ongoing physiological process. Knowledge of the plant growth regulators metabolic pathways can lead to transgenesis or synthesis of a specific inhibitor affecting a step of the pathway in order to increase the yield, prevent the plant from pathogenes etc. Nowadays, the methods of molecular biology play an important role in elucidating metabolic pathways, but still, isolation and identification of new compounds from a plant cannot be omitted.

2 Aims and scopes

There is very little known about cytokinin nucleotides. Although the endogenous occurrence of these compounds in plants has been predicted a decade ago, up to date, their functions are still not fully understood. Interestingly, when applied exogenously, cytokinin nucleotides play a role in animal cell death and are of medicinal interest. The lack of analytical methods for intact cytokinin nucleotides determination together with lack of standards are the main obstacles in understanding of their functions both in plant and animal organism. To study the biological importance of these compounds, new methods are essential.

The main objectives of this doctoral thesis are:

1. Measurement of the intracellular accumulation of *ortho*-topolin riboside-5'-monophosphate (oTRMP) after its exogenous application on T-lymphoblastic leukemia (CEM) cells using capillary electrophoresis.

2. Development, optimization and validation of a mass spectrometry compatible chromatographic method for direct determination of intact cytokinin nucleotides. Application of this method on identification and quantification of intracellular metabolites of isopentenyladenosine (iPR) treated chronic myelogenous leukemia (K562) cells.

3. *In vitro* assaying of the affinity of the recombinant non-secreted CK dehydrogenases (*At*CKX1, *At*CKX3 and *At*CKX7) towards cytokinin nucleotides by capillary electrophoresis.

4. Development, optimization and validation of a capillary electrophoresis method for simultaneous determination of adenosine and cytokinin nucleotides. Application of this method on assaying an *in vitro* reaction catalyzed by the recombinant *At*IPT1 enzyme.

3 Literature review

3.1 Plant hormones

Plant hormones (phytohormones) are essential substances for plant growth and development. For this reason, they are considered as primary metabolites (together with proteins, saccharides, lipids, aminoacids etc.) (Repcak, 2002a). The metabolic pathways are interconnected. Isoprenoids are typically secondary metabolites, but the cytokinin side-chain as well as the molecules of abscisic or gibberelic acids are of isoprenoid origin. On the other hand, secondary metabolites such as caffeine or theobromine originate from the purine biosynthetic pathway which is fundamental e.g. for nucleotides and nucleic acids synthesis. The action site of phytohormones is located either in the same tissue (or even cell) as the biosynthesis is (e.g. ethylen), but more often, they are transported to the effector tissue. From the chemical aspect, they represent a wide group of compounds of various chemical natures. In general, the molecular weight of phytohormones is relatively low (Repcak, 2002b). Plant hormones are present at very low concentrations – typically 0.1 – 50 $ng.g^{-1}$ fresh weight (Ljung et al., 2004). Plant growth and development is controlled by six major phytohormone classes: auxins, cytokinins, gibberellins, abscisic acid, ethylene and brassinosteroids. Biosynthesis, metabolism, signal perception, biological effects and crosstalk of these are still not fully understood and are studied intensively. Besides that, other compounds (e.g. jasmonic acid, peptide hormones etc.) have certain regulatory functions in plants (Repcak, 2002b).

3.1.1 Cytokinins

Cytokinins (CKs) are a group of plant hormones involved in the cell division, the counteraction of senescence, the regulation of apical dominance and the transmission of nutritional signals (Sakakibara, 2004). The first cytokinin – kinetin (K) was discovered by Miller and Skoog in the 1950´s (Miller et al., 1955). Identification of other CKs followed later; zeatin (Z) was isolated from immature endosperm of maize (*Zea mays* L.) by Letham (Letham, 1963b). Naturally occurring CKs are *N6* substituted adenine derivatives. The substitution can be either isoprene-derived or aromatic. They can exist in several metabolic forms: nucleotides, nucleosides and nucleobases (Mok et Mok, 2001). Common endogenous CK free bases are isopentenyladenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (DHZ). Physico-chemical properties as well as the biological functions are affected by the type and degree of (sugar, phosphate, side chain) modifications (Yong et al., 2009). For chemical structures see Fig. 1. Some synthetic diphenylurea derivatives showed CK activity as well (Sakakibara, 2004).

3.1.1.1 Cytokinin biosynthesis

Isoprenoid CKs were identified in tRNA hydrolysates shortly after their discovery (Skoog et al., 1966). It was assumed that hydrolysis of tRNA is a major source of CKs. This assumption was however refused after tRNA turnover calculations. A *de novo* CK biosynthetic pathway was proposed (Klämbt, 1992). On the other hand, degradation of tRNA is a source of *c*Z-type CKs which occurs in significant amounts in some plants such as rice (*Oryza sativa* L.) or maize (*Zea mays* L.). Thus, the hydrolysis of tRNA can contribute to the total CK pool.

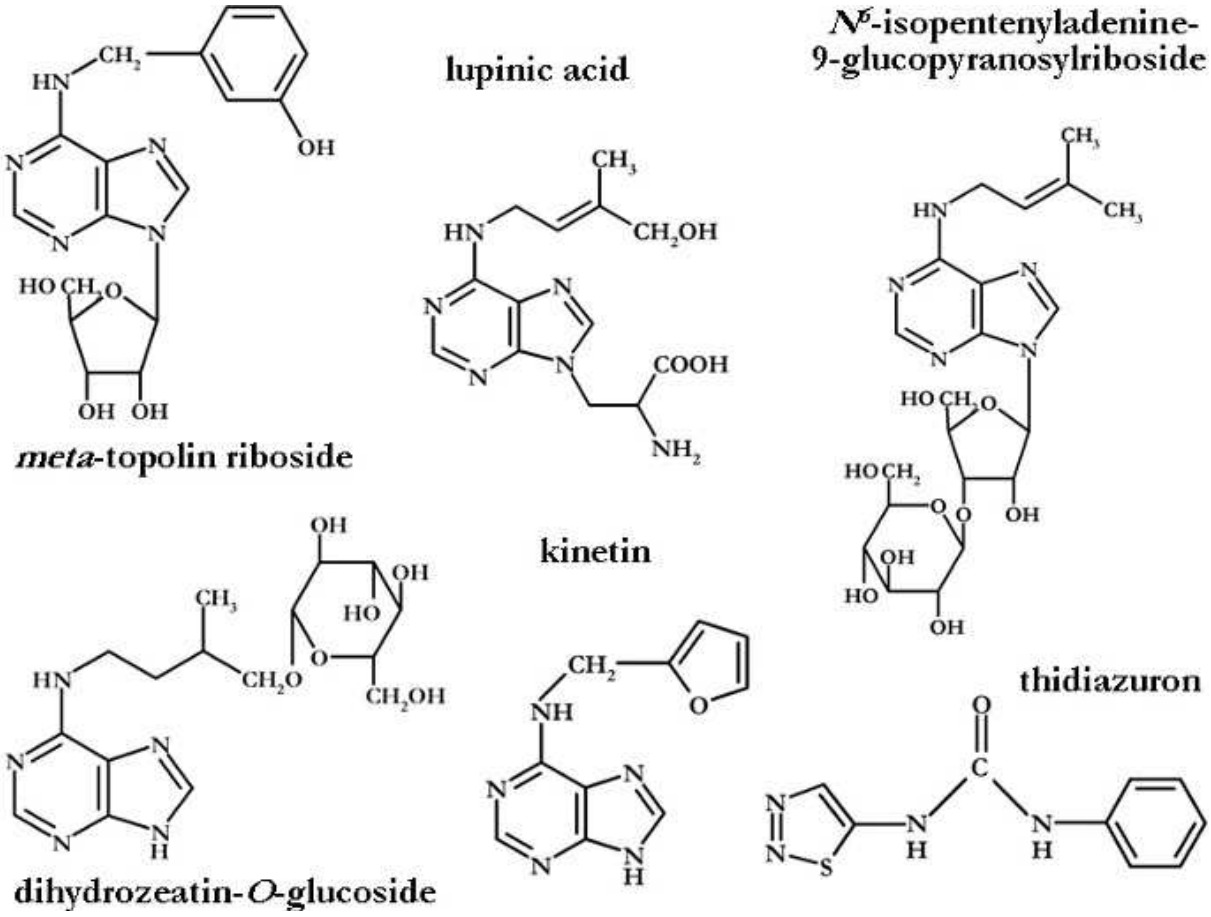

Fig. 1 Structures of representative adenine-type and phenylurea-type cytokinins (adopted from Galuszka et al., 2008).

The discovery of an enzyme capable of isopentenylation of the exocyclic *N6* amino group of AMP was a breakthrough in CK *de novo* biosynthesis. The enzyme was partially isolated from a slime mold (*Dictyostellum discoideum* L.) by Taya and coworkers (Taya et al., 1978). Dimethylallyl-diphosphate (DMAPP) and adenosine-5´-monophosphate (AMP) were converted into isopentenyladenosine-5´-monophosphate (iPMP) by the cell-free extract of this mold. An enzyme with isopentenyltransferase activity encoded by the gene 4 of the Ti-plazmid of *Agrobacterium tumefaciens*, a gram negative bacterium causing the crown gall disease, was later identified as a DMAPP:AMP

isopentenyltransferase (Barry et al., 1984; Akiyoshi et al., 1984). AMP had been considered the only acceptor of DMAPP till 2001, when the sequencing of the *Arabidopsis thaliana* genome was completed. Subsequently, 9 genes encoding isopentenyltransferases (*At*IPT1- *At*IPT9) were identified (Kakimoto 2001, Takei et al., 2001). Recombinant proteins of two of them were studied in detail. Both (*AtIPT1* and *AtIPT4*) showed higher affinity towards ATP and ADP as acceptors of the isopentenyl group. This lead to a conclusion that the first CKs appear to be the di- and triphosphorylated nucleotides and a new scheme of the early stages of CK biosynthesis was proposed (Kakimoto 2001). Isopentenyltransferases have been later identified also in petunia (*Petunia hybrida* L.), hop (*Humulus lupulus* L.) and rice (*Oryza sativa* L.) (Zubko et al., 2002; Sakano et al., 2004; Sakamoto et al., 2006). Substrate specificity of the hop ipts was studied more closely (Chu et al., 2010).

In higher plants, the most abundant and active are the Z-type CKs. iP nucleotide dependent and independent pathways have been suggested. The first mentioned assumes formation of Z-type CKs by hydroxylation of iP nucleotides side-chain catalyzed by P450 monooxygenase. Two genes CYP735A1 and CYP735A2 were recently identified in *A. thaliana* genome (Takei et al., 2004b). Substrate specificity study of these enzymes showed the preference of iPMP and iPDP over iPTP as a substrate for hydroxylation. The iP independent pathway was proposed by Åstot and co-workers (Åstot et al., 2000). According to this hypothesis, the Z is formed directly using a hydroxylated precursor. Such compound, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP), was found in bacteria, but not identified in plants yet (Hecht et al., 2001).

Isoprenoids are synthesized in plants by two independent pathways. Methylerythritol phosphate pathway (MEP) is located in plastids, mevalonate pathway (MVA) in the cytosol. DMAPP, the side-chain donor, can be synthesized by both of them. Experiments with ^{13}C labelling showed that the side-chain of iP as well as of *t*Z are originated from the MEP pathway and the side-chain of *c*Z from the cytosolic MVA pathway. (Kasahara et al., 2004). On the other hand, iPMP-independent pathway derived side-chain is formed in cytosol (Åstot et al., 2000). The presence of *cis-trans* isomerase capable of converting the *c*Z to *t*Z and vice-versa was also reported (Bassil et al., 1993). Figure 2 shows the current scheme of CK biosynthesis. However, experiments with IPT knock-outs do not support its significance, at least in *Arabidopsis thaliana* (Miyawaki et al., 2006).

3.1.1.2 Metabolism of endogenous CKs

From the physiological aspect, CKs can be divided into active, transport and inactivated or storage forms. The highest CK activity appertains to free-bases (Spichal et al., 2004). It is assumed they originate (at least partially) from the phosphoribosyltransferase activity of the cytokinin-activating enzyme, directly from nucleotides – the first products of CK biosynthesis. This gene was found in rice and named the LONELY GUY (LOG) because of the reducted inflorescence phenotype caused by its mutation (Kurakawa et al., 2007). Ribosides are considered to be the transport-form of CKs. Deribosylation to release the nucleobase has not been reported yet (Sakakibara, 2006). N- and O-

glycosylation are the ways of CK inactivation. N-glycosylation occurs at the *N3*, *N7*, *N9* positions of purine and is irreversible. O-glycosylation of the side-chain hydroxyl group on the other hand is reversible and the sugar moiety (glucose, xylose) can be cleaved by β-glucosidase (Brzobohaty et al., 1993). Occurrence of methylthiol substituted (position 2 of the purine ring) CKs is usually connected with bacterial infection (Omer et al., 2004, Pertry et al., 2009).

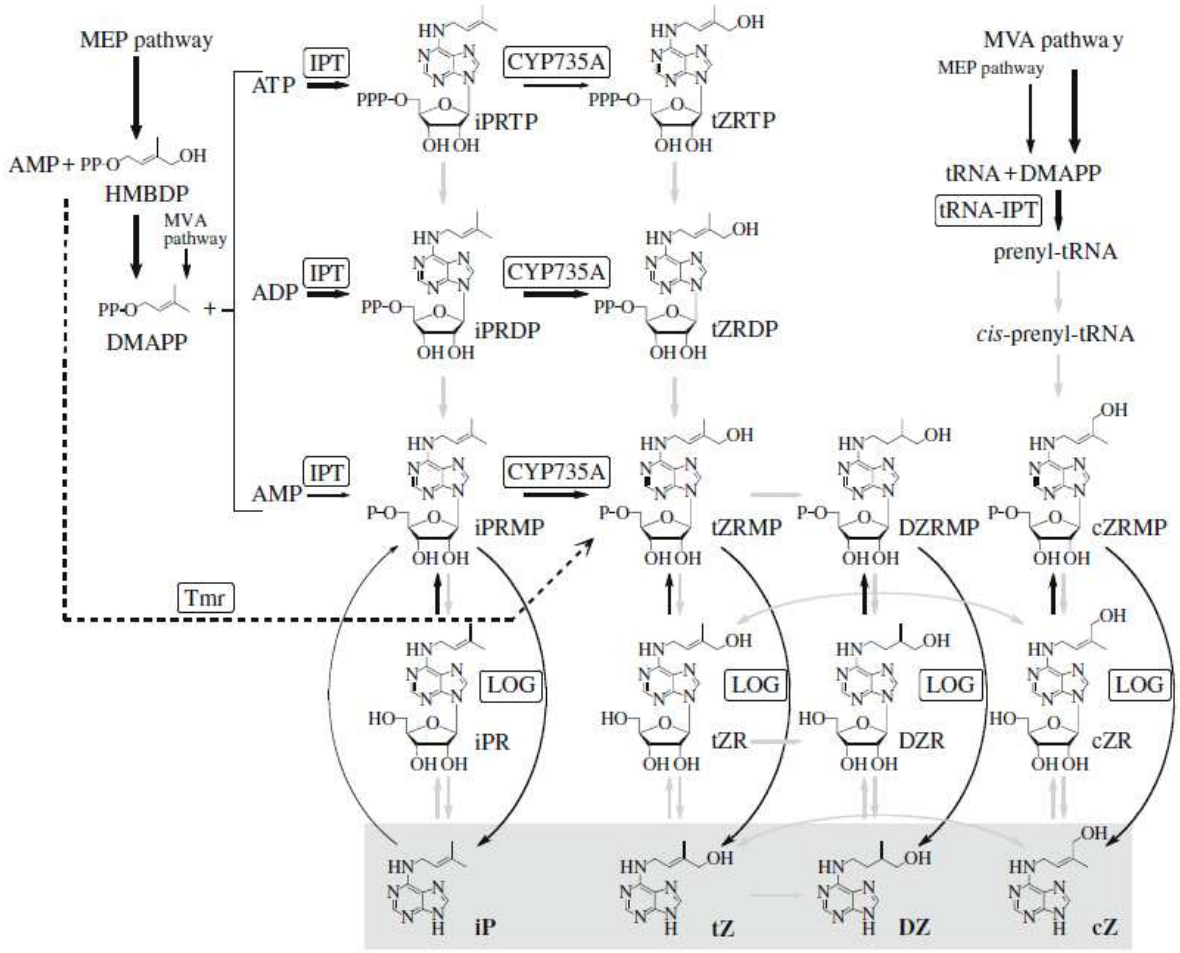

Fig. 2 Current model of isoprenoid cytokinin (CK) biosynthesis pathways in plants. Gray arrows show the steps in which the relspective enzymes have not yet been identified (scheme adopted from Kamada-Nabusada et Sakakibara, 2009).

3.1.1.3 Cytokinin degradation

The degradation of CKs is an important mechanism of inactivation in order to achieve CK homeostasis. The key enzyme involved in this process is cytokinin dehydrogenase (CKX). The first report of its action was brought in 1971 in crude tobacco extracts (Paces et al., 1971). The degradation itself represents inactivation of CKs by oxidative cleavage of its side chain. The genome

of *A. thaliana* contains genes (*AtCKX1 – AtCKX7*) encoding seven isoenzymes (Schmulling et al., 2003). Each isoenzyme has a different substrate specificity (Galuszka et al., 2007; Kowalska et al., 2010). CKXs recognize the double bond of the isoprenoid side chain, thus O-glycosides and DHZ are less affected by their activity (Armstrong, 1994). The most recent findings revealed an affinity of these enzymes towards CK nucleotides (III.).

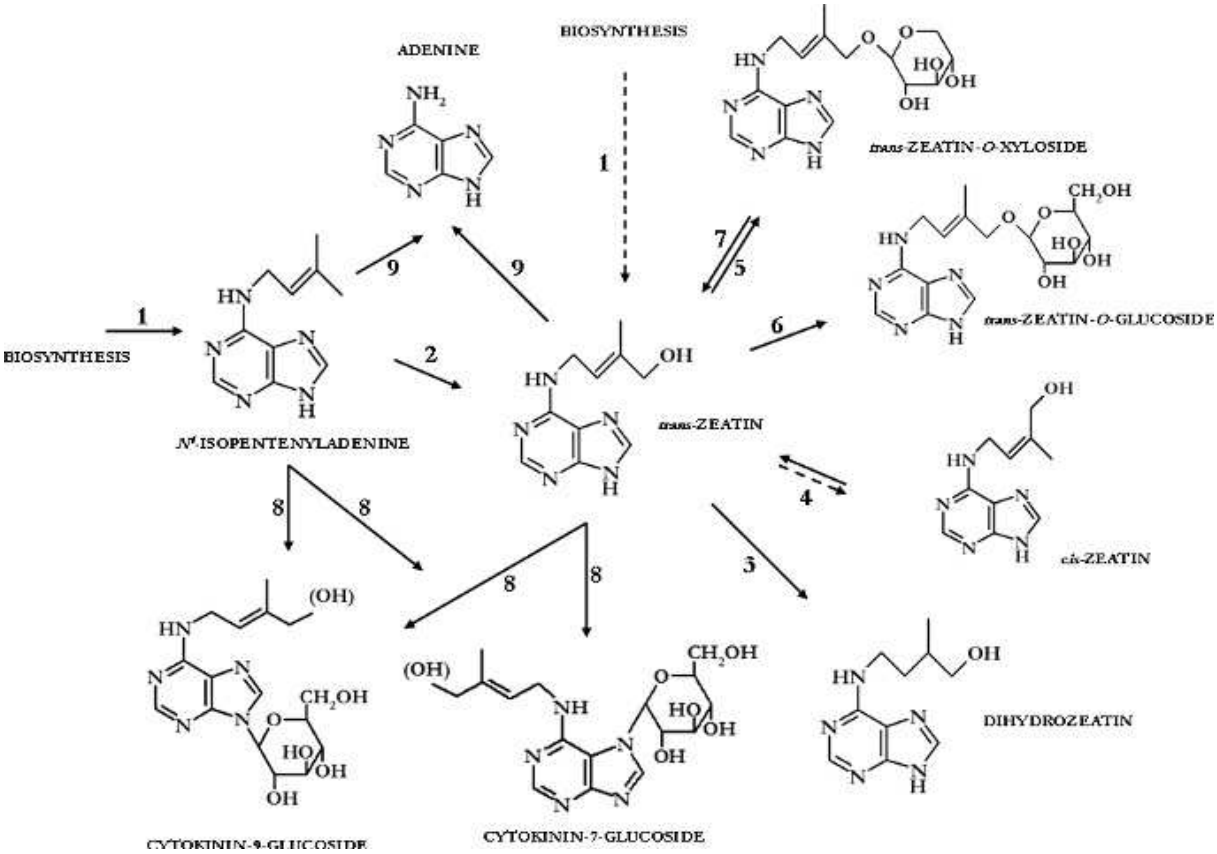

Fig. 3 Basic metabolic pathways of isoprenoid cytokinins. Enzymes participating in individual steps: 1 – adenylate isopentenyltransferase; 2 – cytochrome P-450 monooxygenase; 3 – zeatin reductase; 4 – zeatin isomerase; 5 - zeatin-O-glucosyltransferase; 6 - zeatin-O-xylosyltransferase; 7 - β-glucosidase; 8 – cytokinin-N-glucosyltransferase; 9 – cytokinin dehydrogenase. Dashed line indicates only predicted metabolic pathway, which have not been proved yet (scheme adopted from Galuszka et al., 2008)**.**

3.1.1.4 Biological activity and benefits for humans

Aside from the functions in plants, CKs have interesting effects also in animal cells. Isopentenyladenosine (iPR), kinetin riboside (KR) and 6-benzylaminopurine riboside (BAPR) were found to be inhibitors of the proliferation of myeloid leukemia cells. Free bases of these compounds

did not exhibit cytotoxic effect, but they induced differentiation of the HL-60 cells. The cell death was accompanied by rapid ATP depletion (Ishii et al., 2002).

Two mechanisms of purine-mediated cell death induction are assumed. For the first one, an intracellular phosphorylation is essential to trigger the apoptosis (Lin et al., 1988; Cottam et al., 1993), the second is mediated via extracellular adenosine receptors (Kohno et al., 1996). It was suggested that the mechanism of action of CK nucleosides is closely related to adenosine metabolism, which requires phosphorylation by adenosine kinase (Ishii et al., 2002). Later, it was confirmed that conversion of CK ribosides into corresponding mononucleotides via adenosine kinase pathway is essential in cell death induction in HL-60 cells (Mlejnek et Dolezel, 2005).

Synthetic analogues of CK ribosides were synthesized, characterized and tested successfully against various cancer cell lines (Dolezal et al., 2007). A broad spectrum of naturally occuring CKs (including nucleobases, glucosides, ribosides, nucleotides of isoprenoid as well as aromatic character) were assayed for antiproliferative activity on a diverse panel of 59 cell lines. This comprehensive study revealed strong cytotoxicity of *ortho*-topolin riboside. CK nucleotides (mono- di- and triphosphorylated) showed activity very similar to their respective ribosides, indicating extracellular dephosphorylation and subsequent intracellular re-phosphorylation (I.).

3.2 Analytical methods for identification and quantification of phytohormones

Altering the levels of phytohormones is connected with various physiological processes in plant growth and regulation, e.g. the increase of ethylene concentration is involved in fruit ripening, elevation of abscisic acid level is related to drought or salt stress. Differences in CK levels between a mature tissue and a meristematic one is obvious and well described (Repcak, 2002b).

The phytohormone level in non-transgenic plants is usually very low, typically, it does not exceed 50 g g^{-1} fresh weight. This, together with the matrix complexity and the phytohormone metabolites structure similarity (including positional and optical isomers) demands not only a detection system sensitive enough to detect trace amounts, but also a highly efficient separation and sufficient purification (Ljung et al., 2004).

Basically, there are two major approaches in phytohormone analysis. The first one is focused on screening of a number of metabolites of a phytohormone class (e.g cytokinin profiling) (Novak et al., 2008). This approach is suitable for studying of the biosynthesis, metabolism and degradation of a phytohormone. Separation of isomers is fundamental in this case. The second approach is based on analysis of the most important (active) form of each phytohormone class and is suitable e.g. for hormone cross-talk studies (Kojima et al., 2009). Because different hormone groups vary in their chemical nature, simultaneous extraction and purification are the main challenge (Ljung et al., 2004).

The first attempts to quantify a known growth regulator or to identify a new one with similar (or better) activity were performed by bioassays. The activity of a compound is evaluated by an extent of

the biological response (Tarkowski et al., 2004). The principle of assaying CK activity can be based on e.g. stimulation of tobacco callus growth, retention of chlorophyll in excised wheat leaves or dark induction of betacyanin synthesis in Amaranthus cotyledons (Holub et al., 1998). Plant extract is a complex mixture of compounds. Fractionation (most often by means of chromatography) in order to separate the analytes of interest is therefore needed prior to detection (radioimmunoassay – RIA, enzyme-linked immunoassay - ELISA or the recently most used one – MS – mass spectrometry) (Tarkowski et al., 2004).

3.2.1 Liquid chromatography

It has been over a hundred years since Mikhail Semyonovich Tsvet – a russian botanist – first separated chlorophylls on a column filled with calcium carbonate. He named it chromatography. The significance of his discovery was not fully understood then. His work was followed in the 30s of the 20th century, but the real expansion came after the Second World War. Nowadays, chromatography is the third most used analytical technique (right behind weighting and pH adjustment) (Svec 2009).

Chromatography is a separation method where the analytes are distributed between two phases. The first one is stationary, the mobile phase flows through in one direction. In case of liquid chromatography, the mobile phase (eluent) is a fluid. The sample is injected into the mobile phase and is forced through the separation column filled with the stationary phase. The components of the sample are more or less retarded by the interactions with the stationary phase and are eluted by the mobile phase to the detector. The order of analytes is characterized by retention time (t_R) which is specific for the given conditions (Ettre, 1993; Ardrey, 2003).

3.2.1.1 High performance liquid chromatography (HPLC) in CK analysis

The first attempt to analyze CKs by means of chromatography (paper and thin layer chromatography – PC, TLC) came in 1973 (Hewett, 1973). This type of chromatography is not common nowadays, however it still can be used, especially in experiments, where a phytohormone is applied exogenously to a plant or a cell suspension culture to study the *in vivo* conversion. For this purpose, radioactively labelled compounds are often utilized with the advantage of sensitive detection (Taverner et al., 1999).

The demand of high sample throughput requires an approach capable of automatization. The vast majority of methods, these days, thus uses HPLC. Diverse polarity of CKs allows the separation on a reversed phase (RP) stationary phases. The mechanism of the analytes retention is the hydrophobic interaction between the non-polar stationary phase (usually C18) and the analyte. CKs

are eluted gradually by increasing the percentage of an organic modifier (methanol – MeOH or acetonitrile - ACN) in the mobile phase.

Typically, the diameter of a column used for CK separation is 4.6 or 2.1 mm (Redig et al., 1996b, Novak et al., 2003). However, it was showed that the strategy of using miniaturized columns (diameter of 1 or 0.3 mm) can significantly improve the sensitivity. Lower sample and mobile phase consumption are also the advantages of this approach (Prinsen et al., 1998, Nordstrom et al., 2004a).

Ultra-high performance liquid chromatography (UHPLC) represents the current state-of-art of the chromatography techniques. Columns packed with sub-2-μm particles, high flow-rate of the mobile phase and instrumentation able to withstand the pressure over 400 bar significantly improve resolution, increase peak capacity and reduce the analysis time and the consumption of the organic modifier (Novak et al., 2008).

3.2.2 Mass spectrometry

Mass spectrometry is an analytical technique for molecular mass determination. Among other analytical tools it has an outstanding position. Although the first studies of J.J. Thomson date back to the beginning of the 20th century (1912), the most rapid progress was made during the last decade. Coupling the MS with gas chromatography in 1958 and liquid chromatography in 1985 can be considered as the milestones of separation techniques – MS detection hyphenation. The recent applications are mainly proteome and metabolome oriented (Hoffman et Stroobant, 2007).

The mass spectrometer consists of an ion source, an analyser and a detector. The first mentioned serves to form ions from neutral molecules. A variety of ionization techniques are available (electron ionization – EI, chemical ionization – CI, matrix assisted laser desorption ionization – MALDI etc.). Nowadays, the most frequently used one is the electrospray ionization – ESI. An advantage of this technique over previously mentioned ones is the ability of easy coupling to HPLC and succesfull ionization of molecules with wide range of polarity and molecular mass. Ions are extracted into the analyser, where they are separated according to mass to charge (*m/z*) ratio. The type of analyser used depends on the application requirement. Time of flight (TOF) instruments are suitable for exact mass determination, ion trap (IT) mass spectrometers are able to perform MS^n analyses, etc. Finally, the ions reach the detector, where they are detected and converted into an electric signal proportional to their abundance. The mass analyser and the detector (sometimes also the ion source) are evacuated to avoid any unwanted collisions with the air molecules (Hoffman et Stroobant, 2007).

3.2.2.1 Mass spectrometry in CK identification and quantification

Although the identification of CKs by MS (coupled to gas chromatography – GC) was reported already in 1977 (Moris, 1977), the first HPLC-MS method came later (Yang et al., 1993). Since then, MS coupled to LC became the method of choice for CK structure elucidation as well as quantification. The main reason is the labour and time intensive derivatization step needed prior to GC (Tarkowski et al., 2004). A variety of ionization techniques were successfully used in the early days of CKs LC-MS, including atmospheric pressure chemical ionization (APCI) (Yang et al., 1993) and fast atom bombardment (FAB) (Astot et al., 1998). The most common one remained ESI (Novak et al., 2003). The detection limits of the early methods allowed to measure the cytokinin content in gram amounts of plant tissue or to analyze a transgenic plant tissue with high cytokinin levels (Tarkowski et al., 2004).

Mass analysers most often utilized in CK quantitative analysis comprise single quadrupole (Q) (Novak et al., 2003) and tripple quadrupole (QqQ) (Prinsen et al., 1998). The use of a double-focusing magnetic sector, IT and Q-TOF for this purpose was also reported (Astot et al., 1998; Chen et al., 2010; Fletcher et al., 2007). The latter mentioned is preferably applied in qualitative analysis (identification of new CKs) by determination of the exact mass (Dolezal et al., 2007). The quantitative analysis by selected ion monitoring (SIM) mode (Novak et al., 2003) or multiple reaction monitoring (MRM) mode (Novak et al., 2008) is usually performed by the isotope dillution method. This method requires the addition of an internal standard (IS – represented by a stable isotope labelled phytohormone) during extraction. Despite the sample losses throughout the purification, the labelled:unlabelled compound ratio stays unchanged. The amount of endogenous compound is calculated in the end (Ljung et al., 2004).

3.2.3 Capillary electrophoresis in phytohormone analysis

Capillary electrophoresis (CE) is an analytical technique used to separate compounds using a conductive liquid (usually aqueous) medium (background electrolyte – BGE; running buffer) under the influence of an electric field. The separation is based on differences in their charge-to-size ratios and takes places in a (typically fused silica) capillary. The migration of the analytes is caused by the electrophoretic mobility of the charged molecules in the electric field and the elecotroosmotic flow (EOF) which is generated on the negatively charged inner capillary wall (Baker, 1995). Although phytohormones are usually charged molecules (auxins, abscisic, giberellic acid) and can be separated relatively easily, CE is not used for analysis of plant regulators widely. The main reason is most likely lower sensitivity of this analytical tool compared to chromatographic methods. The volume of the separation capillary is usually in low microliter range, allowing only nanoliter-scale sample injection volume. Despite the main drawback mentioned above, CE was used several times for analysis of phytohormones. IAA was determined succesfully by CE with UV detection by Liu and Li (Liu et Li, 1996). A real sample consisted of 30 g of Miss Vanda Joaquim (a hybrid orchid cultivar) shoot apices.

Several partitioning steps were needed prior to CE measurment itself. Olsson et al. took advantage of electroactivity of the IAA and its conjugate with aspartic acid (IAAsp) and developed a CE separation with amperometric detection. The method was used to determine two auxins in pea (*Pisum sativum* L.) shoot apices and stems (0.75 g FW) after extraction followed by SPE desaltation and in pea xylem sap, which was injected into the capillary directly (Olsson et al., 1996). Native fluorescence of indoles allowed also the use of fluorescence detector to analyze these compounds (Olsson et al., 1998b). Due to lower sensitivity of the lamp induced fluorescence compared to laser induced fluorescence (LIF) and also to amperometric detection, higher amount of plant material (3 – 10 g FW of pea) had to be used. On the other hand, the only sample pretreatment needed was filtration. The potential of CE with fluorimetric detection to analyze auxins in plant material was demonstrated also by Brüns and co-workers (Brüns et al., 1997). An amount of 10 g FW per sample of maize, castor bean and kalanchoe (*Zea mays* L.*, Ricinus communis* L., *Kalanchoë daigremontiana* Hamet et Perrier) was used to determine endogenous levels of IAA after one-step SPE procedure. A plant hormone profiling method by micellar electrokinetic chromatography (MEKC) was developed by Liu and co-workers (Liu et al., 2002a). Although a complete separation of seven phytohormones (including representatives of cytokinin, auxin, gibberellin class and abscisic acid) was achieved and a large volume sample stacking (LVSS) approach applied successfully, the method was capable to detect only the level of ABA in a transgenic tobacco plant flowers. A CE-LIF method for ABA determination in was reported by Liu and co-workers (Liu et al., 2003). The detection sensitivity allowed to measure the ABA concentration in a crude extract of 1g FW of tobacco leaf. In order to analyze ABA by LIF, a derivatization step (labelling with 8-aminopyrene-1,3,6-trisulfonate) had to be applied prior to separation. Partial filling (PF) MEKC methods with MS detection were developed to screen for the presence of gibberellines in coconut milk. Separation of the analytes was performed using permanent (Ge et al., 2007) or dynamic (Ge et al., 2008a) cationic capillary coating to reverse the electroosmotic flow. GA_1 and GA_3 were identified in coconut water by both methods.

3.2.3.1 Capillary electrophoresis in CK analysis

In 1997, a method for CK analysis utilizing CE was reported for the first time (Pacaková et al., 1997). Acidic and alkaline buffers were compared as BGEs. Acetone extracts of 9 g of plant material (wheat, sugar beet and tobacco) were extracted (liquid-liquid extraction) to purify the sample. The presence of ZR and Z were detected by CE-UV subsequently. Acidic conditions were preferred, as less interferences were observed. CKs were studied using CE-UV by Bartak and co-workers (Bartak et al., 1998; Bartak et al., 2000c; Bartak et al., 2000d). Although no plant material was analysed by these methods, the interactions of CKs with cyclodextrines were described and some important physico-chemical constants of CKs were determined. Various endogenous isoprenoid (iP, Z, DHZ, tZR, tZOG, DZOG, tZMP) and aromatic (oT, K, KR) CKs were identified in coconut milk by CE-MS (Ge et al., 2004; Ge et al., 2005; Ge et al., 2006a; Ge et al., 2006b). Multistep sample clean-up was needed prior

to the CE-UV and CE-MS measurement. Reversed phase SPE and mixed-mode ion exchange (cation for CK bases and glucosides; anion for the nucleotides) was needed to pre-concentrate the analytes. Despite the massive development of CE in last decades (hyphenation with MS detection in particular), chromatographic methods are still more suitable for CK analysis in a diverse plant material. CE however, when applied in peculiar cases such as determining physicochemical parameters of CKs or assaying *in vitro* enzymatic reactions, can be used with an advantage. This topic was reviewed comprehensively by Tarkowski and co-workers, recently (Tarkowski et al., 2009).

4 Material and methods

4.1 Chemicals

- The standards of CK nucleotides were synthesized partially in the Laboratory of Growth Regulators (CK monophosphates) and in BIOLOG (Bremen, Germany) (CK di- and triphosphates). The details of the preparation are described in the Supplements part (I., II.).

- All the chemicals used were at least of analytical-reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA); Merck (Darmstadt, Germany); Lach-Ner (Brno, Czech Republic) and OlChemIm (Olomouc, Czech Republic).

- Deionised (Milli-Q) water was obtained from Simplicity 185 (Millipore, Bedford, MA, USA) or from a Sartorius Atrium 611 UV water purification system (Göttingen, Germany).

4.2 Biological material

- The cultivation, treatment and harvesting of CEM and K-562 cells are described in detail in the Supplements section (I., II.).

- The recombinant non-secreted CK dehydrogenases (*At*CKX1, *At*CKX3, *At*CKX7) were isolated and purified as described in the Supplements section (III.).

- The isolation and purification of the *At*IPT1 enzyme is described in the Supplements section (IV.).

4.3 Equipment

- Alliance 2690 Separations Module (Waters, Milford, MA, USA) linked simultaneously to a PDA 996 (Waters) and a ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface (Micromass, Manchester, UK) was used in HPLC-MS experiments (II.).

- An Acquity UPLC® System (Waters, Milford, MA, USA) combined with Quattro micro API (Micromass, Manchester, UK) was used in HPLC-MS/MS experiments (II.).

- An Agilent^{3D} CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a UV absorbance DAD was used in CE-UV experiments (I., III., IV.). For the CE-MS measurements, the CE instrument was coupled to an Esquire 3000 plus ion trap MS (Bruker Daltonics, Bremen, Germany) via a CE-MS interface from Agilent (IV.).

- The HPLC-QqTOF-MS experiment was performed on an HP1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a microQtof II (Bruker Daltonics, Bremen, Germany) mass spectrometer (IV.).

4.4 Methods

- Extraction of nucleotides was adopted from Friedecky et al. (2007). The trichloroacetic acid extracts were back-extracted with diethylether twice and analyzed immediately (I). The extracts were freeze-dried and reconstituted in the initial mobile phase for LC-MS measurements (II).

- The quantification of endogenous nucleotides was performed according to a slightly modified method of Friedecky et al. (2007). Briefly, the nucleotides were separated as anions in negative separation mode at 25 kV in a 82.5 cm total; 72 cm effective lenght; 75 μm i.d. bare-fused capillary (Polymicro Technologies, Phoenix, AZ, USA). Aqueous 40 mM citrate and 0.8 mM CTAB (cetyltrimethylammonium bromide) adjusted to pH 4.3 with GABA (γ-aminobutyric acid). Quantification was done using corrected peak areas at detection wavelength 254 nm. The identification of ATP, ADP, AMP and oTRMP was based on UV spectra comparison and by spiking with the standard solutions. The data were processed in ChemStation software (I.).

- To assay the affinity of non-secreted recombinant *At*CKX enzymes towards CK nucleotides, separations were carried out in a bare-fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). Total length of the capillary was 64.5 cm (56 cm effective), 50 μm i.d. Glycine/ammonia (100 mM) BGE at pH 10.0 was used and the analytes were separated as anions in positive mode. Separations were carried out at 30 kV, the capillary cassette was thermostated to 20 ℃. All quantifications were done at 268 nm using corrected peak areas and uridine-5´-monophosphate as an internal standard. The data were processed in ChemStation software (III).

- The separations for the purpose of isopentenyltransferase activity assay were performed in a 75 μm i.d.; 375 μm od; 80.5 cm (72 cm effective length) bare-fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). Aqueous 100 mM sarcosine/ammonia at pH

10.0 BGE. The separations were run at a constant voltage of 30 kV. The capillary cartridge was thermostated to 20℃ and the detection wavelength was 254 nm. The data were processed in ChemStation software. The CE-MS experiments were carried out using 30 mM ammonium formate as the BGE and a 50 μm i.d. capillary. Other parameters were the same as mentioned for CE-UV experiments. The sheath liquid was constantly driven into the ion source at a flow rate of 0.3 ml/h by a syringe pump (74900 series, Cole-Parmer, Vernon Hills, IL, USA). The analytes were detected in ESI(-) mode. The MS parameters were set as follows: spray voltage 1.5 kV, probe temperature 250℃, sheath gas (nitrogen) 2 psi, dry gas (nitrogen) 4 l/min. CE–MS data were analyzed using Bruker Daltonics Data Analysis software (IV.).

- RP Symmetry C18 column (150×2.1 mm, 5 μm; Waters) was used to determine the chromatographic purities of the synthesized monophosphates standards. The analytes were separated using a mobile phase consisting of 2 % B (MeOH) for the first 10 min, followed by a linear gradient to 90 % B from 10 to 25 min then isocratic 90% B from 25 to 35 min and finally a linear gradient back to 2 % B from 35 to 45 min, balanced throughout by (A) aqueous 15 mM ammonium formate pH 4.0, at a constant flow rate of 0.25 ml/min. Using post-column splitting (1:1), the effluent was introduced simultaneously into the DAD detector (scanning range 210–400 nm, with 1.2-nm resolution) and the MS detector (source temperature 100 ℃, capillary voltage −3.0 kV, cone voltage −30 V, desolvation temperature 250 ℃). Nitrogen was used both as the desolvation gas (600 l/h) and cone gas (50 l/h). HPLC purities were determined from DAD chromatograms (II.).

- For HPLC-MS/MS identification of intracellular metabolites of iPR in treated K-562 cells (II.) RP column (Gemini-NX, 150×2 mm, 3 μm; Phenomenex, Torrance, CA) was used. The analytes were separated using a mobile phase consisting of 2% B (MeOH) for the first 2 min, followed by a linear gradient to 65 % B from 2 to 25 min, then isocratic 90% B from 25 to 35 min, and finally re-equilibration with 2% B from 35 to 45 min, balanced throughout by (A) aqueous 20 mM acetic acid adjusted to pH 10.0 with ammonium hydroxide, at a constant flow rate of 0.2 ml/min. ESI(−) MS/MS conditions were set as follows: source temperature 100 ℃, capillary voltage −5.0 kV, cone voltage −33 V, collision energy 25 eV, and desolvation temperature 350 ℃. Nitrogen was used as the desolvation gas (400 l/h). The mass spectrometer was operated in product ion scan mode (II.).

- Method validation, intracellular metabolite quantification and the stability experiment were performed under chromatographic conditions used for intracellular metabolite identification (see above). The effluent was directly introduced into the electrospray source, and the analytes were quantified by negative ESI(−) selected ion monitoring (SIM) analysis of the deprotonated $[M–H]^-$ ion. Analyte concentrations were calculated by the isotope dilution method. Due to the lack of deuterium-labelled standards, to quantify different nucleotides, the

corresponding *trans*-zeatin phosphates tracers were used. The column was thermostatically maintained at 20 ℃ to maximize the stability of th e retention times (II.).

- The chromatographic conditions used in reaction products HPLC-QqTOF-MS identification were adopted from (II.). The effluent was introduced to the ion source of a microQtof II (Bruker, Bremen, Germany). The QqTOF was operated in negative ESI(-) MS fullscan mode (*m/z* 50 - 1500), using a capillary voltage of 3.5 kV; end plate offset -500V; dry gas (nitrogen) temperature 200℃, flow 6 l/min and the nebulizer g as pressure 20.3 psi. Experimental molecular mass values were compared with those deduced from the formula. As a measure of fidelity, the difference between both values was calculated as follows: (experimental mass – calculated mass) / calculated mass x 10^6 (ppm) (IV.).

5 Survey of results

- In order to study the intake mechanism of oTRMP (and other CK phosphates as well) by cancer cells, CE measurements of cell extracts were performed. The method of Friedecký (Friedecky et al., 2007) was utilized after slight modifications. The experiments revealed that the 5'–phosphates of oTR (oTRMP, oTRDP and oTRTP) are dephosphorylated extracellularly, transported into the cells as ribosides and then re-phosphorylated intracellularly. The simultaneous application of the adenosine kinase inhibitor A-134974 in concentration of 5 $\mu mol.l^{-1}$ prevented CEM and K 562 cells from intracellular oTRMP accumulation and the subsequent cell death. The obtained results indicate a similar operation mode of CK nucleotides as fludarabine monophosphate or tricribine monophosphate (Malspeis et al., 1990; Wotring et al., 1986) (I.).

- A HPLC method for baseline separation of nine isoprenoid CK nucleotides was developed and optimized. The buffer contained only a volatile MS compatible salt (20 mM ammonium acetate; pH = 10.0) and alowed complete separation of all the studied compounds including three pairs of *cis* – *trans* zeatin nucleotide isomers. Ionization of the analytes was achieved in negative ESI mode. Peak efficiency and t_R were thoroughly monitored during the optimization and validation with the respect to the column robustness and showed to be satisfactory. The developed method showed linearity ($R^2 \geq 0.995$) in the concentration range of 1 – 1000 pmol. The recovery varied between 70 and 97% and the analytical accuracy between 61 and 96%. The detection limits were in high femtomole levels. The ionization efficiency of the analytes decreased with the increasing number of phosphate groups. The method was applied to identify the intracellular metabolites of iPR in K 562 cells by means of tandem mass spectrometry as iPMP, iPDP and iPTP (II.).

- The affinity of the recombinant non-secreted *Arabidopsis thaliana* cytokinin dehydrogenases (*At*CKX1, *At*CKX3 and *At*CKX7) was determined by CE. The iP-type CK nucleotides were better substrates for the studied *At*CKX enzymes than the Z-type ones. Vacuolar *At*CKX enzymes (*At*CKX1 and *At*CKX3) degraded iPDP and iPTP, in preference over iPMP. The decrease of Z-type nucleotides was less significant. Same pattern was observed for the mitochondrial *At*CKX7 with even more pronounced preference for iP-type nucleotides over Z-type ones and a more rapid advance (III.).

- A CE-based assay for *At*IPT1 isopentenyltransferase activity determination was developed, optimized and validated. Separation of six nucleotides (ATP, ADP, AMP, iPTP, iPDP, iPMP) was achieved within 20 minutes with high theoretical plate number (up to 400 000). Aqueuos 100 mM sarcosine/ammonia buffer at pH 10.0 was used as the BGE. The detection limits reached, are in low micromolar levels. The method showed acceptable repeatability of the migration times (within 1.3%) as well as of the peak areas (within 1.8%) and linearity ($R^2 \geq$

0.99982) in the concentration range of 5 – 1000 μmol.l^{-1}. The possibility of direct online iP-type nucleotides identification by CE-MS was investigated. Due to significant differences in ionization efficiency of iPMP, iPDP and iPTP, the idenfication of the *in vitro* reaction product was performed by CE-UV and HPLC-QqTOF-MS. The product was unequivocally identified as iPDP with a mass accuracy of -5.06 ppm. (IV.).

6 Conclusions and perspectives

This thesis describes the development of HPLC-MS and CE-UV/MS methods for identification and quantification of intact cytokinin nucleotides. The methods were applied in analysis of these compounds in leukemia cells and *in vitro* enzymatic reaction mixtures. It is concluded that:

- Intracellular CK nucleotides formation is essential in cell death triggering in K 562 and CEM cells treated with the corresponding CK riboside or nucleotide.

- Intact mono- , di- and triphosphorylated Z and iP-type nucleotides can be determined by an RP-HPLC method with single/tandem MS detection.

- iPMP, iPDP and iPTP are the intracellular metabolites of iPR in K 562 leukemia cells. iPMP is the most abundant one.

- Non-secreted *At*CKX enzymes have affinity towards both iP and Z-type cytokinin nucleotides.

- Capillary electrophoresis is a suitable analytical technique to assay the *in vitro* reaction catalyzed by the recombinant *At*IPT1 and this approach may bring a new light into the early steps of CK biosynthesis.

In summary, new methods for CK determination by HPLC and CE were developed. For the first time, they are able of simultaneous analysis of mono- di- and triphosphorylated CK nucleotides. With their help, the CK biosynthesis can be studied in more detail. Furthemore, the mechanism of the CK cytotoxicity against cancer cell lines can be further explored.

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