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Biological effects and metabolism of *cis*-zeatin-type cytokinins in plants

PhD Thesis

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Statement

I hereby declare that the work presented in this thesis is my own and was carried out entirely with help of literature and aid cited in the thesis. This thesis is not a subject of any other defending procedure.

Prague, Czech Republic

July 28th, 2011 Mgr. Silvia Gajdošová

On behalf of the co-authors of the papers published, I hereby confirm the agreement with inclusion of the papers below into the dissertation thesis of Silvia Gajdošová. The papers were produced as a team work and the particular contribution of Silvia Gajdošová is specified at the beginning of appendix of the thesis.

Prague, Czech Republic

July 28th, 2011 Ing. Václav Motyka, CSc.

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Abbreviations

Ade - adenine

Ado - adenosine

AHK - Arabidopsis histidine kinase

AHP - Arabidopsis histidine phosphotransfer protein

ARR - Arabidopsis response regulator

AVG - L-α-[(2-aminoethoxy)vinyl]glycine

BAP - N^6-benzyladenine

CHASE - cyclases/histidine kinases associated sensory extracellular

*cis*Z - *cis*-zeatin

*cis*Z7G - *cis*-zeatin 7-glucoside

*cis*Z9G - *cis*-zeatin 9-glucoside

*cis*ZOG - *cis*-zeatin O-glucoside

*cis*ZR - *cis*-zeatin 9-riboside

*cis*ZRMP - *cis*-zeatin 9-riboside-5´-monophosphate

*cis*ZROG - *cis*-zeatin 9-riboside O-glucoside

CK - cytokinin

CKX - cytokinin oxidase/dehydrogenase

CRE1 - cytokinin response 1

Cys - cysteine

D - aspartate

DHZ - dihydrozeatin

DHZ7G - dihydrozeatin 7-glucoside

DHZ9G - dihydrozeatin 9-glucoside

DHZOG - dihydrozeatin O-glucoside

DHZR - dihydrozeatin 9-riboside

DHZRMP - dihydrozeatin 9-riboside-5´-monophosphate

DHZROG - dihydrozeatin 9-riboside O-glucoside

DMAPP - dimethylallyl diphosphate

FAD - flavin adenine dinucleotide

FAD - flavin adenine dinucleotide

H – histidine

HMBDP - 1-hydroxy-2-methyl-2(E)-butenyl-4-diphosphate

iP - isopentenyladenine

iP - N^6-(2-isopentenyl)adenine

iP7G - N^6-(2-isopentenyl)adenine 7-glucoside

iP9G - N^6-(2-isopentenyl)adenine 9-glucoside

iPR - N^6-(2-isopentenyl)adenine 9-riboside

iPRMP - N6-(2-isopentenyl)adenine 9-riboside-5´-monophosphate

IPT - isopentenyltransferase

LOG - lonely guy

MEP - methyl-D-erythritol 4-phosphate

MeS-*cis*ZR - 2-methylthio-*cis*-zeatin riboside

MVA - mevalonic acid (mevalonate)

NAA - α-naphtalene acetic acid

SAM - S-adenosyl methionine

Ti - tumor-inducing

*trans*Z - *trans*-zeatin

*trans*Z7G - *trans*-zeatin 7-glucoside

*trans*Z9G - *trans*-zeatin 9-glucoside

*trans*ZOG - *trans*-zeatin O-glucoside

*trans*ZR - *trans*-zeatin 9-riboside

*trans*ZRMP - *trans*-zeatin 9-riboside-5´-monophosphate

*trans*ZROG - *trans*-zeatin 9-riboside O-glucoside

WOL - wooden leg

Summary

Cytokinins (CKs) are plant hormones that affect a wide range of developmental processes. The most important group of isoprenoid CKs represent zeatin and its derivatives occurring in two, *cis* and *trans,* positional isomers. Whereas *trans*-zeatin (*trans*Z) was found to be a highly bioactive substance, *cis*-zeatin (*cis*Z) has been viewed for years as inactive or weakly active adjunct to its corresponding *trans* counterpart playing only an insignificant physiological role in plants.

The occurrence of *cis*Z-type CKs was found in a great number of plant species with especially high levels identified in species of family *Poaceae.* All tested derivatives of *cis*Z, surprisingly including also zeatin-N9-glucoside, delayed dark-induced chlorophyll degradation in oat and wheat leaf segments. Additionally, *cis*Zs effectively induced cell division in CK-dependent tobacco callus. The most pronounced activity was exhibited by *cis*Z riboside (*cis*ZR) in the two types of CK bioassays. Metabolism of both zeatin isomers differed in short-term as well as in long-term experiments, which was supported also by various affinity of CK degrading enzyme, CK oxidase/dehydrogenase (CKX), to individual *cis* and *trans* isomers. Primary root enlargement of *Arabidopsis* seedlings was inhibited by *cis*ZR in the same or similar way as by *trans*ZR, presumably via restriction of auxin (NAA) response. On the other hand, *cis*ZR might have impaired chlorophyll accumulation in *Arabidopsis* in the presence of auxin by an ethylene-dependent route different from *trans*ZR. Phenotypic analysis of mutants in *cis*Z biosynthesis, lacking tRNA-isopentenyltransferases (AtIPT2 and AtIPT9) showed chlorotic changes, growth retardation and alterations in leaf and seed shape in *atipt2 9* double mutant. However, according to the obtained data these defects might be caused predominantly by defective protein translation because of lacking tRNA prenylation and significant decrease of isopentenyladenine derivatives rather than by *cis*Z deficiency.

With respect to the latest knowledge of *cis*Z accumulation in tissues exhibiting restricted growth and under stress conditions as well as considering the data presented here it is hypothesized that *cis*Z may function in plants under growth limiting conditions to preserve a minimal CK response necessary for their survival.

Súhrn

Cytokiníny (CKs) sú rastlinné hormóny, ktoré ovplyvňujú celý rad vývojových procesov. Najdôležitejšou skupinou izoprenoidných CK predstavuje zeatín a jeho deriváty, ktoré sa vyskytujú v dvoch, *cis* a *trans*, pozičných izoméroch. Zistilo sa, že zatiaľ čo *trans*-zeatín (*trans*Z) je biologicky vysoko aktívna látka, *cis*-zeatín (*cis*Z) bol odjakživa považovaný za neaktívny alebo málo aktívny v porovnaní s jeho *trans* náprotivkom a že má vo fyziológii rastlín len zanedbateľnú úlohu.

Výskyt CKs *cis*Z typu bol popísaný u veľkého počtu rastlinných druhov, a ich vysoké hladiny boli detegované najmä u zástupcov triedy *Poaceae*. Všetky testované deriváty *cis*Z, prekvapivo vrátane zeatín-N9-glukozidu, účinne spomaľovali degradáciu chlorofylu v tme v listových segmentoch ovsa a pšenice. Okrem toho, *cis*Zs účinne indukovali bunkové delenie u kalusu CK-dependentného tabaku. Najvýraznejšiu aktivitu spomedzi derivátov *cis*Z v oboch typoch CK biotestov vykazoval *cis*Z ribozid (*cis*ZR). Metabolizmus izomérov zeatínu sa líši v krátkodobých aj dlhodobých experimentoch, čo bolo podporené aj rôznymi afinitami CK degradačného enzýmu, CK oxidázy / dehydrogenázy (CKX) k jednotlivým *cis* a *trans* izomérom. Rast primárneho koreňa klíčencov *Arabidopsis* v prítomnosti auxínu (NAA) bola inhibovaná *cis*ZR pravdepodobne rovnakým alebo podobným spôsobom ako v prípade *trans*ZR, prostredníctvom obmedzenia auxínovej odpovede. Na druhej strane, aplikácia *cis*ZR viedla k zníženiu akumulácie chlorofylu u *Arabidopsis* v prítomnosti auxínov podľa všetkého etylén-závislou cestou avšak odlišnou od *trans*ZR. Fenotypová analýza mutantov v biosyntéze *cis*Z, chýbajú im tRNA izopentenyltransferázy (AtIPT2 a AtIPT9), odhalila chlorotické zmeny, spomalenie rastu, zmeny tvaru listu a semien u *atipt2 9* dvojitého mutanta. Avšak, na základe získaných údajov tieto zmeny by mohli byť spôsobené skôr postihnutou transláciou bielkovín, pretože chýba prenylovaná tRNA, a výrazným znížením izopentenyladenínových derivátov než nedostatkom *cis*Z.

S ohľadom na posledné publikované poznatky o akumulácii *cis*Z v tkanivách vykazujúcich obmedzenie rastu a v učitých podmienkach stresu, ako aj s ohľadom na údaje uvedené v tejto práci predpokladáme, že *cis*Z by mohol fungovať v rastlinách za okolností, ktoré obmedzujú rast, aby sa zachovala minimálna CK odpoveď nevyhnutné pre ich prežitie.

1 Introduction

Plants are sesile organisms that have been for ages in the centre of human attention. Original harvesters focusing on planting and providing better nutrition and care to plants and thus became farmers. Further knowledge based on observations and on the first empirical agricultural experiments has led to improvements of crop quality including yield. Last century in plant science has been in spirit of elucidation of biochemical mechanisms underlying plant growth and development. It may provide the solutions to assure higher food production as only better understanding will allow us to obtain sufficient and quality fare with minimal impact on environment heading to the sustainable and prosperous civilization.

Astonishing variability of different forms in plant kingdom is the result of complex interactions among plant predispositions, genotype, and changing environmental conditions. Plants respond to the environment by modulation of their growth and development. They perceive exogenous stimuli and convert them into internal signal bearing molecules which are not directly involved in metabolism and/or development but are able to alter these processes at low concentrations. Intensity and nature of environmental signals must be interpreted by plant very precisely and accurately to preserve its existence and to allow further propagation. Therefore, plants possess several groups of molecules, plant hormones, which determine and alter the plant habitus. It has been known for a long time that interaction between two phytohormones, auxin and cytokinin, is important for outcome of organogenesis (Skoog and Miller, 1957). Recent research proved auxin as inducer of new organ formation and cytokinins (CKs) as substances able to modulate it (Pernisova *et al.*, 2009; Shani *et al.*, 2010). CKs comprise wide range of compounds including *cis*-zeatin (*cis*Z) and its derivatives with significant impact on plant physiology related to food production. As the information about *cis*Z-type CKs is very limited and identification of components allowing higher quality yields is enormously needed it was decided to study this group of CKs.

2 Aims and scope

This PhD thesis stems from the suppositions that *cis*-zeatin (*cis*Z) and its derivatives are more prevalent and probably more relevant to cytokinin (CK) biology than previously considered. The main objective was to reveal the distribution of the *cis*Z-type CKs within the plant kingdom, to determine their biological activities and metabolic pathways in selected plant species and formulate hypothesis concerning physiological significance of *cis*Z. Following partial goals have been outlined:

- To select suitable experimental plant material for studying the *cis*Z biology;
- To assign biological activities of various *cis*Z derivatives in selected CK bioassays;
- To describe metabolism of *cis*Z in chosen plant species;
- To test potential effects of *cis*Z-type CKs in further extending experiments depending on the outcome of so far obtained data;
- To utilize tRNA-IPT mutants with a putative *cis*Z deficiency to search for *cis*Z specific phenotype;
- To formulate hypothesis about *cis*Z function *in planta.*

3 Literature review

3.1 Cytokinins

3.1.1 Discovery and definition of cytokinins

The first factor facilitating cell division in the presence of auxin was identified in heated DNA preparations from herring sperm and named kinetin (Miller *et al.*, 1955). The whole group of compounds exhibiting the same or similar activity in bioassays as kinetin was designated kinins (Miller *et al.*, 1955; Miller *et al.*, 1956). As the same term had been formerly used for animal-related substances with different properties, the name has been changed into cytokinins (CKs). It was proposed that only structural purine derivatives as kinetin, zeatin and N^6-benzyladenine etc. with kinetin-like biological activity of promoting cell division in plants represent group of CKs (Skoog *et al.*, 1965). Later on, some synthetic aromatic derivatives of urea were found also to exhibit CK activities (Halmann, 1990), however, they are not classified as "true" CKs.

3.1.2 Chemical structure of purine CKs and their activity

CKs are phytohormones which encompass a group of N^6 substituted adenines with their side chain moiety of either aromatic or isoprenoid nature (Riefler *et al.*, 2007; Zažímalová *et al.*, 1999; Letham and Palni, 1983).

3.1.2.1 Aromatic CKs

Structure of aromatic CKs is essentially involving aromatic side chain as benzyl in case of N^6-benzyladenine (BAP) or furyl moiety of kinetin (Miller *et al.*, 1955). Although kinetin was at the beginning of CK era, hydroxylated form of BAP, N^6-(*ortho*-hydroxybenzyl)adenosine which was identified in poplar (Populus x robusta) as the first native aromatic CK 10 years after isoprenoid zeatin (Horgan *et al.*, 1973). Later on, the whole group of BAP derivatives hydroxylated in *ortho*-, *meta*, and *para*- positions gained its trivial name according to a Czech equivalent for the Populus – topol (Strnad *et al.*, 1997). Aromatic CKs are minor constituents of CK pool in plants (Saenz *et al.*, 2003) but high activity of *meta*-topolin in CK bioassays is comparable to those of *trans*Z (Holub *et al.*, 1998; Kaminek *et al.*, 1987). Comprehensive screens of various BAP derivatives with different phenyl ring substituents revealing high biological activity but inability to interact with CK receptors for most of them (Doležal *et al.*, 2007; Dolezal *et al.*, 2006).

3.1.2.2 Isoprenoid CKs

Isoprenoid CKs rise from basic structure of N^6-(2-isopentenyl)adenine (iP; Figure 1) and their variability is due to modifications of N^6 side chain moiety. However, the first plant CK of isoprenoid character was not iP itself but its hydroxylated form, zeatin, found in immature kernels of maize (*Zea mays*), which inspired its name (Letham, 1963). Zeatin shows high bioactivity in bioassays therefore is considered to be the most important isoprenoid CK (Skoog and Armstrong, 1970). Presence of double bond and a hydroxyl moiety in zeatin side chain allows formation of two positional isomers *cis* and *trans* (Figure 1). The two zeatin isomers differ in their bioactivity as *cis*-zeatin (*cis*Z) exhibits very low CK effects in contrast to *trans*-zeatin (*trans*Z) (Schmitz *et al.*, 1972). Saturation of double bond in zeatin side chain yields another CK, dihydrozeatin (DHZ), showing lower biological activity (Schmitz *et al.*, 1972).

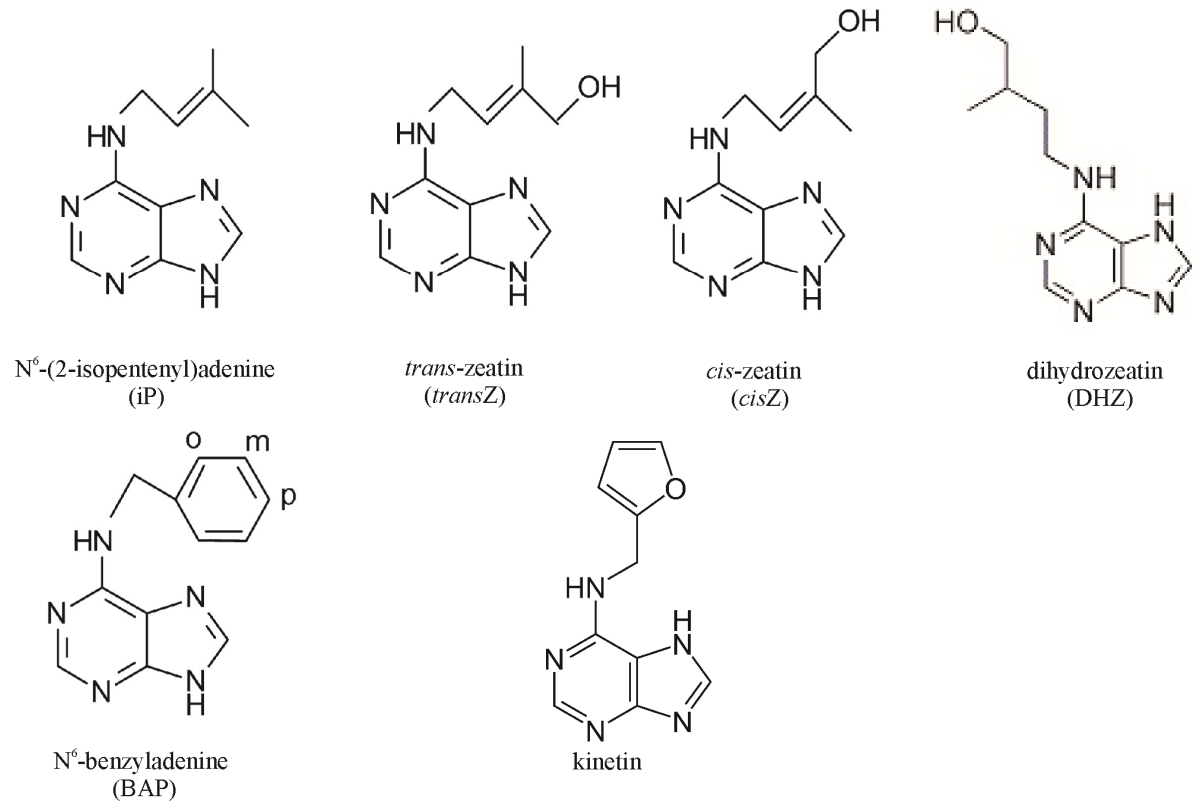


Figure 1: Structures of isoprenoid (upper row) and aromatic cytokinins (lower row).

3.1.2.3 Zeatins

Zeatins (Figure 1) belong among the most common isoprenoid CKs in plants. One of the two positional isomers, *trans*Z was firstly detected in maize tissues extracts (Letham, 1963;

Letham *et al.*, 1964) whereas *cis*Z was isolated from soluble RNA (tRNA) of maize, spinach and garden peas afterwards (Hall *et al.*, 1967). Further research elucidated that *cis*Z is a constituent of leucyl-tRNA in peas (Einset *et al.*, 1976). Later on, *trans*Z has been shown to occur in high amounts in plants and as a free CK is the most active isoprenoid CK in most bioassays in contrast to *cis*Z (Schmitz *et al.*, 1972). Because of weak *cis*Z bioactivity, little attention was paid to its occurrence or abundance. Existing historical data depict *cis*Z and its derivatives in plants as a tRNA constituent with very low biological activity (Schmitz *et al.*, 1972; Kamínek *et al.*, 1979). For that reason, *cis*Z-type CKs have been overlooked in the past and has been considered as adjunct to CK homeostasis with none or insignificant biological impact. More detailed inspection of *cis*Z CKs *in planta* revealed that some species accumulate *cis*Zs as predominant CKs e.g. chickpea and lupine (Emery *et al.*, 1998; Emery *et al.*, 2000) and the increase of *cis*Z CKs levels can be site specific (Mauk and Langille, 1978). These and many other published results subvert the previous image of *cis*Z and suggest that it may play some, so far unidentified, role in physiology of plants.

3.1.3 Biosynthesis of CKs

According to the current knowledge, isoprenoid CKs can be synthesized by two routes on the basis of adenine origin in the CK molecule. The one is tRNA-dependent pathway in which adenine comes from tRNA (Figure 2) and the second, tRNA-idependent, utilizes adenine in form of adenosine-5'-mono-, di-, triphosphate (AMP, ADP, ATP, Figure 3).

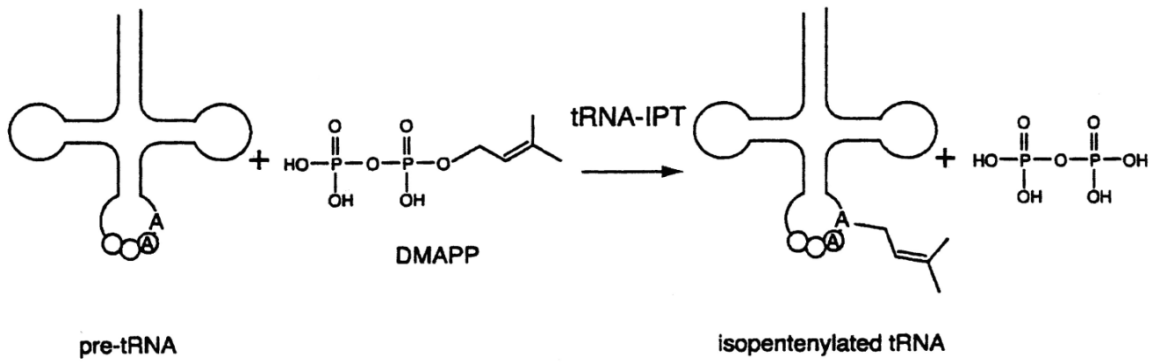


Figure 2: tRNA-dependent CK biosynthesis (Sakakibara, 2004).

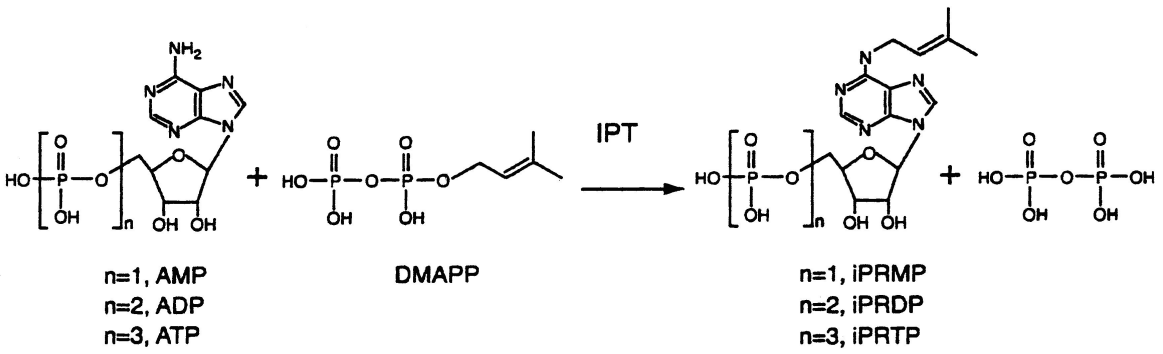


Figure 3: tRNA-independent CK biosynthesis (Sakakibara, 2004).

3.1.3.1 Origin of side chain precursors for CK biosynthesis

Origin of CK side chain precursors is known just for isoprenoid CKs, but there is a hypothesis that aromatic chain precursor might rise from phenolics biosynthesis (Strnad, 1997). Isoprenoid moieties can be supplied either from a cytosolic mevalonate (MVA) pathway or plastidic methyl-D-erythritol 4-phosphate (MEP) route (Kuzuyama, 2002). Whereas the MVA pathway is known to produce the isoprenoid precursors isopentenyldiphosphate and dimethylallyl diphosphate (DMAPP), the MEP route generates additionaly to DMAPP also 1-hydroxy-2-methyl-2(E)-butenyl-4-diphosphate (HMBDP). HMBDP is utilized especially by *Agrobacterium* (Krall *et al.*, 2002).

3.1.3.2 CK biosynthetic enzymes

Initial idea that CKs are synthesized via tRNA degradation was based on the iP presence in tRNA (Skoog *et al.*, 1966). Additional evidence was given by feeding labeled mevalonic acid, cytosolic precursor of DMAPP, to tobacco pith tissue because the radioactivity was localized in iP bound in tRNA (Chen and Hall, 1969). Later on, the tRNA IPT activity was found in *Escherichia coli*, *Lactobacillus acidophillus* or *Zea mays* (Rosenbaum and Gefter, 1972; Holtz and Klambt, 1975, 1978) indicating functioning of tRNA CKs in wide spectrum of organisms.

Discovery of tRNA-independent CK biosynthetic pathway was associated with slime mold *Dictyostelium discoideum* (Taya *et al.*, 1978). The study revealed that partially purified enzyme preparation exhibited isopentenyltransferase (IPT) activity as it utilized DMAPP but different source of adenine, 5'-AMP, as reaction substrate for CK formation.

3.1.3.3 Identification of CK biosynthetic genes

Although the initial evidence of IPT activity was detected in tobacco, the first CK biosynthetic gene was identified in *Agrobacterium tumefaciens*, the pathogenic bacterium capable of infecting the plant and causing genesis of unorganized tumors (Gelvin, 1990). Tumor morphology is affected by three genes *tmr, tzs* and *tms* present in Ti (tumor-inducing) plasmid. While the *tmr* and *tms* genes are localized in T-DNA, the *tzs* is a part of vir-region of the Ti-plasmid, outside of T-DNA (Garfinkel *et al.*, 1981). It was later described that mutation of *tmr* gene causes formation of root teratomas while defects in *tms* lead to formation of shoot-like teratomas (Morris, 1986). In 1983, Akiyoshi *et al.* (1983) found out that insertion of transposone into *tmr* locus reduced levels of *trans*Z and *trans*ZR, which led to formation of of root-producing tumor. Based on this finding, the authors proposed that tumor morphology may result from altered phytohormonal balance. A year later, the *tmr* gene was proven to encode DMAPP : AMP dimethylallyltransferase (later renamed as isopentenyltransferase) responsible for CK biosynthesis (Akiyoshi *et al.*, 1984).

Further advance in elucidation of CK biosynthetic genes was achieved after sequencing of *Arabidopsis thaliana* genome (Kaul *et al.*, 2000). New source of knowledge allowed to screen 8 novel sequences in *Arabidopsis* (AtIPT1-8) similar to *tmr* isopentenyltransferase (IPT) of *Agrobacterium* (Takei *et al.*, 2001), and almost simultaneously an additional gene AtIPT9 (Kakimoto, 2001) with the lowest homology to other *Arabidopsis* IPTs. Unlike other IPTs, AtIPT9 together with AtIPT2 resembled DMAPP: tRNA-isopentenyltransferase (Kakimoto, 2001). Assays of substrate specificities of AtIPTs revealed surprising affinity of AtIPT4 to ADP and ATP comparing with AMP-preferring IPT enzymes of *Agrobacterium* and *Dictyostelium discoideum* (Taya *et al.*, 1978; Akiyoshi *et al.*, 1984; Kakimoto, 2001).

3.1.3.4 Formation of CK bases

After addition of the isoprenoid side chain moiety, either from MVA or MEP pathway, to pre-*t*RNA catalysed by corresponding tRNA IPTs resulting in formation of CK nucleotides (Figure 2). In case of tRNA-independent pathway AMP or ADP/ATP is employed (Figure 3). Subsequent cleavage of ribose-5'-monophosphate and release of free bioactive CK bases are enabled by phosphoribohydrolase LONELY GUY (LOG) (Kurakawa *et al.*, 2007). Current model of isoprenoid CK biosynthesis in *Arabidopsis thaliana* is presented in Figure 4.

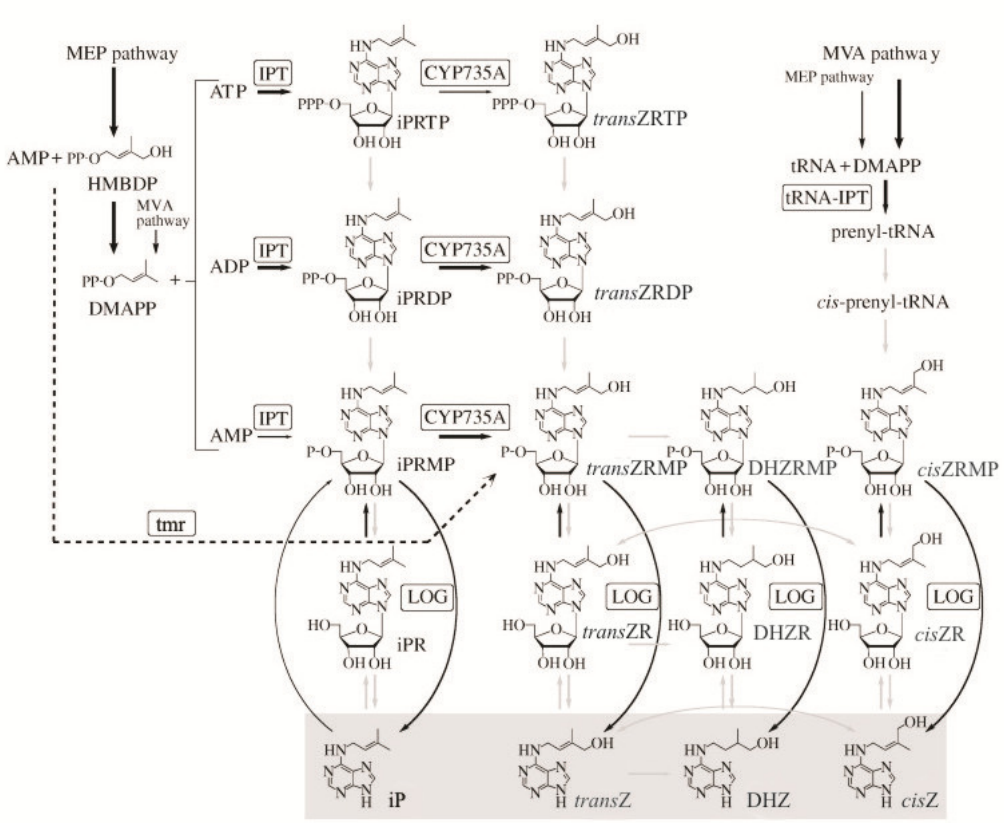


Figure 4: CK biosynthesis. (Kamada-Nobusada and Sakakibara, 2009). Abbreviations of CKs - iP, N^6-(2-isopentenyl)adenine; iPR, N^6-(2-isopentenyl)adenine 9-riboside; iPRMP, N6-(2-isopentenyl)adenine 9-riboside-5´-monophosphate; *trans*Z, *trans*-zeatin; *trans* ZR, *trans*-zeatin 9-riboside; *trans*ZOG, *trans*-zeatin O-glucoside; *trans*ZROG, *trans*-zeatin 9-riboside O-glucoside; *trans*ZRMP, *trans*-zeatin 9-riboside-5´-monophosphate; *cis*Z, *cis*-zeatin; *cis*ZR, *cis*-zeatin 9-riboside; *cis*ZOG, *cis*-zeatin O-glucoside; *cis*ZROG, *cis*-zeatin 9-riboside O-glucoside; *cis*ZRMP, *cis*-zeatin 9-riboside-5´-monophosphate; DHZR, dihydrozeatin 9-riboside; DHZOG, dihydrozeatin O-glucoside; DHZROG, dihydrozeatin 9-riboside O-glucoside; DHZRMP, dihydrozeatin 9-riboside-5´-monophosphate. Abbreviations of enzymes – isopentenyltransferase (IPT); phosphoribohydrolase LONELY GUY (LOG); tmr-bacterial isopentenyltransferase.

3.1.4 Biosynthesis of cisZ

The most detailed characterization of *cis*Z biosynthesis (Figure 5) was published for its methylthiolated form in bacteria *Salmonella typhimurium* (Persson *et al.*, 1994). It was shown, that *(a)* tRNA-IPT miaA (Connolly and Winkler, 1989) prenylates adenosine moiety in *t*RNA yielding isopentenyladenosine (iPR), then *(b)* sulfurtransferase miaB (Esberg *et al.*, 1999) and methyltransferase miaC modify the adenine ring by addition of methyl and thio group and finally *(c)* the side chain is *cis*-hydroxylated by miaE (Persson and Bjork, 1993; Mathevon *et al.*, 2007). The process runs under aerobic conditions and forms 2-methylthio-*cis*-zeatin riboside (MeS-*cis*ZR), which is released after degradation of tRNA. A minor pathway yielding *cis*ZR was proposed to be exerted by miaE in the presence of oxygen with available isopentenyladenosine (iP) (Persson *et al.*, 1994).

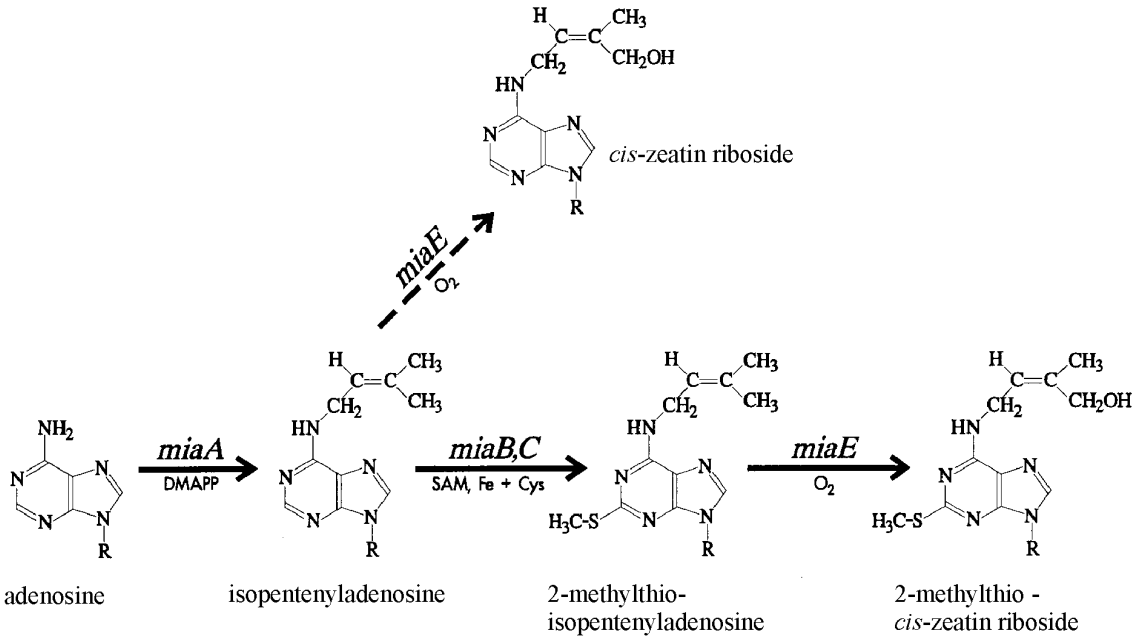


Figure 5: Biosynthesis of MeS-*cis*ZR in *Salmonella typhimurium*. Cys – cysteine; DMAPP - dimethylallyl diphosphate; miaA – tRNA isopentenyltransferase; miaB – sulfurtransferase; miaC – methyltransferase; miaE - monooxygenase; SAM - S-adenosyl methionine (Persson *et al.*, 1998).

In contrast to bacteria, biosynthesis of *cis*Z in plants is scarcely described and solely *Arabidopsis,* maize and rice tRNA IPTs have been identified on the basis of similarities to bacterial tRNA IPT miaA gene. *Arabidopsis* genome comprises of AtIPT2 and AtIPT9, rice possesses two respective homologs OsIPT9 and OsIPT10 and maize has a single ZmIPT10 (Sakamoto *et al.*, 2006; Miyawaki *et al.*, 2006). Whereas AtIPT2 and OsIPT9 share more similarities with AMP IPTs; AtIPT9, ZmIPT10 and OsIPT10 are more distant, resembling prokaryotic tRNA IPTs (Sakamoto *et al.*, 2006; Kakimoto, 2003). Sequence of AtIPT2 harbours zinc finger-like motif C-X_2-C-$X_{12,18}$-H-X_5-H (Takei *et al.*, 2001) and may play a role in tRNA binding (Golovko *et al.*, 2000). It also contains putative HY5 (LONG HYPOCOTYL5) binding region (Song *et al.*, 2008). HY5 is a basic leucine zipper (bZIP) transcription factor that activates photomorphogenesis and root development in *Arabidopsis* (Song *et al.*, 2008).

According to the present knowledge, *cis*Z biosynthesis depends solely on tRNA IPTs in *Arabidopsis*. Evidence was given by Miyawaki *et al.* (2006), who proved that knocking out of both *Arabidopsis* tRNA IPTs, AtIPT2 and AtIPT9, results in complete diminishment of *cis*Z and its derivatives. Cytoplasmic subcellular localization of AtIPT2-GFP indicated possible utilization of DMAPP derived from cytosolic MVA pathway. This assumption was supported by observation that large portion of *cis*Z derivatives contained radiolabelled moiety after [^{13}C]MVA feeding and suggests that tRNA prenylation occurs in the cytosol (Kasahara *et al.*,

2004). However, contribution of plastid MEP pathway to *cis*Z biosynthesis cannot be excluded since large fraction of [^{13}C] originating from labelled 1-deoxy-D-xylulose was detected in isoprenoid side chain of *cis*Z CKs. and these levels are far behind the hypothetical exchange of isoprenoid precursors between MVA and MEP pathway (Kasahara *et al.*, 2004). Existence of plant *cis*Z forming hydroxylase is predicted in spite of the fact that a protein sequence similar to miaE has not been found in *Arabidopsis* genome (Kaminska *et al.*, 2008; Sakakibara, 2006).

3.1.5 Metabolic conversions of CKs

Modifications of CKs can affect either N^6-side chain or adenine moiety (Figure 6). Each type of modification alters CK properties whether it is solubility, resistance to degradation etc. and therefore contributes to diversity of CK activity and availability for various physiological processes in plants as indicated by results of CK bioassays with various CK derivatives.

3.1.5.1 Modifications of adenine moiety

Adenine moiety provides several sites for modifications. It can be methylthiolated in N2 position and N-glycosylation can occur in N3, N7 and N9 positions (Letham *et al.*, 1978). Position N9 is also a site for modification with alanine, ribose and ribosylphosphate (Sakakibara, 2006). Hydroxylated isoprenoid side chain can be O-glucosylated or O-xylosylated.

Methylthiolation at C2 is catalyzed by miaB in prokaryotic organisms (Esberg *et al.*, 1999) and might be plausibly executed by miaB homologous enzyme in *Arabidopsis* (Kaminska *et al.*, 2008) but direct biochemical evidence is missing.

First documented glucosylated form of CKs was reported for radish (*Raphanus sativus*) after feeding with zeatin and further analysis confirmed its identity as zeatin-7-glucoside (Parker *et al.*, 1972; Parker and Letham, 1973). Partially purified enzyme preparation with N7-glucosyltransferase activity was described later (Entsch *et al.*, 1979) and finally Hou *et al.* (2004) identified 2 *Arabidopsis* genes coding for N-glucosyltransferases, UGT76C1 and UGT76C2, responsible for glucosylation of CKs at the N7 and N9 positions. Formation of N7- or N9-glucoside may depend on availability of particular tautomer of purine moiety (Broo and Holmen, 1996).

Glucose moiety may also be attached at position N3 and it is known that the glucose of N3-glucosides can be hydrolyzed by β-glucosidase activity in maize by Zm-p.60.1 (Brzobohaty *et al.*, 1993).

Another modification is alanylation in position N9, but its physiological significance is not understood (Entsch *et al.*, 1983).

Important modification of CKs is ribosylation in position N9. The ribosylation usually confers to CKs some resistance to degradation (Bilyeu *et al.*, 2001; Galuszka *et al.*, 2007), improves CK solubility and reduce CK activity.

Phosphorylation and phosphoribosylation represent other significant CK metabolic pathways, because exogenously applied CKs were found to be readily converted into corresponding nucleotides or nucleosides e.g. in *Phaseolus vulgaris* (Sondheimer and Tzou, 1971). Such interconversions usually involve enzymes common to purine metabolism (Chen, 1997).

Any CK specific kinase has not been described untill now, but several enzymes of adenine metabolism show low but significant affinities to CKs. Therefore, they may contribute to the recycling and interconversions of CK nucleobases. They are represented by two isoforms of adenine phosphoribosyltransferase enzymes, APT2 and APT3 (Allen *et al.*, 2002) which can modify iP or *trans*Z and another enzyme, adenosine kinase ADK2, has been described to phosphorylate iP (Moffatt *et al.*, 2000).

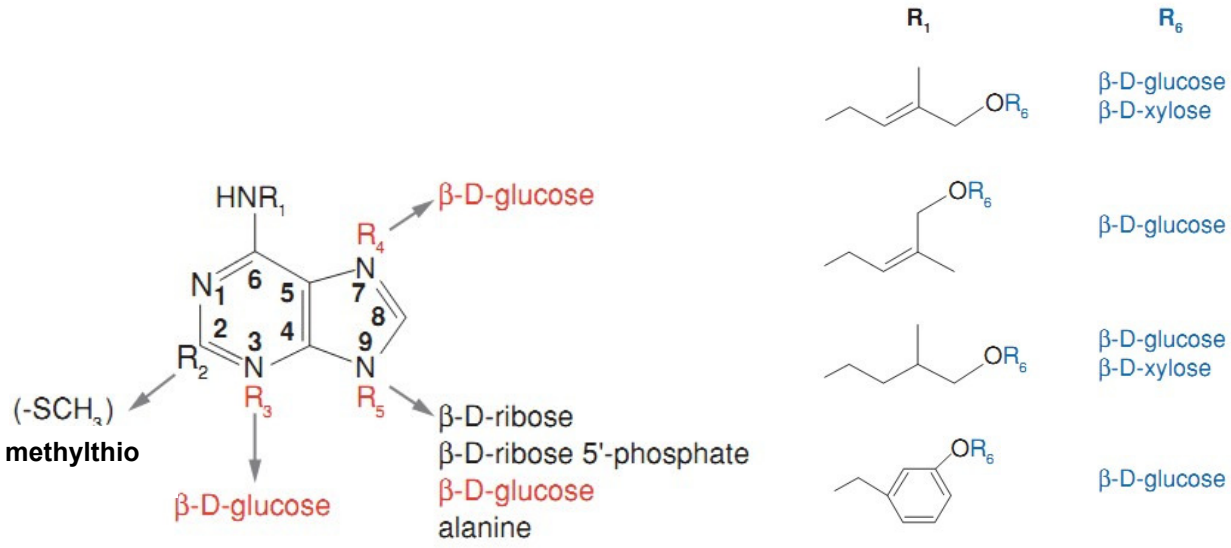


Figure 6: Schematic picture of possible CK modifications (Sakakibara, 2006)

3.1.5.2 Modifications of side chain

Isoprenoid side chain of CKs can be hydroxylated saturated and the hydroxylated CKs may undergo further addition of saccharides.

Cytochrome P450 monooxygenases, CYP735A1 and CYP735A2, hydroxylate side chain of isopentenyladenine nucleotide, which results in formation of corresponding zeatin nucleotide (Takei *et al.*, 2004).

The side chain of *trans*-zeatin can be then reduced by zeatin reductase thus dihydrozeatin is formed. The enzyme was partially purified and characterized (Martin *et al.*, 1989; Gaudinová *et al.*, 2005).

Hydroxyl group of zeatin can occur in *cis* or *trans* position and interconversion of isomers may be executed by *cis-trans* isomerase (Bassil *et al.*, 1993). The isomerase requires dithiothreitol, flavin and light for the reaction. However, no further characterization has been performed and there are only scarce reports of *cis-trans* isomerase activity found *in vivo*.

Enzyme with O-xylosylation activity was purified from embryos of *Phaseolus vulgaris.* It recognized UDP-xylose and dihydrozeatin (DHZ) and *trans*Z but not *cis*Z as substrates (Turner *et al.*, 1987). Embryos of the related species, *Phaseolus lunatus*, were found as a rich source of O-glucosyltransferase, which utilized UDP-glucose and *trans*Z but did not accept DHZ or *cis*Z (Dixon *et al.*, 1989). Corresponding genes, ZOX1 and ZOG1, were isolated later on (Martin *et al.*, 1999a, b). Glucosyltransferases specific for *cis*Z, *cis*ZOG1 and *cis*ZOG2, were identified in maize with highest expression in roots and kernels and roots, respectively (Martin *et al.*, 2001; Veach *et al.*, 2003)

3.1.5.3 CK degradation

Degradation of CKs was first demonstrated by cell-free enzyme system from tobacco callus tissue (Paces *et al.*, 1971). Afterwards the cytokinin oxidase/dehydrogenase (CKX) activity was detected in a number of plant materials (Armstrong, 1994). The CKX selectively cleaves unsaturated N^6 isoprenoid side chain of CKs and releases free adenine (adenosine) plus side-chain derived aldehyde (Figure 7).

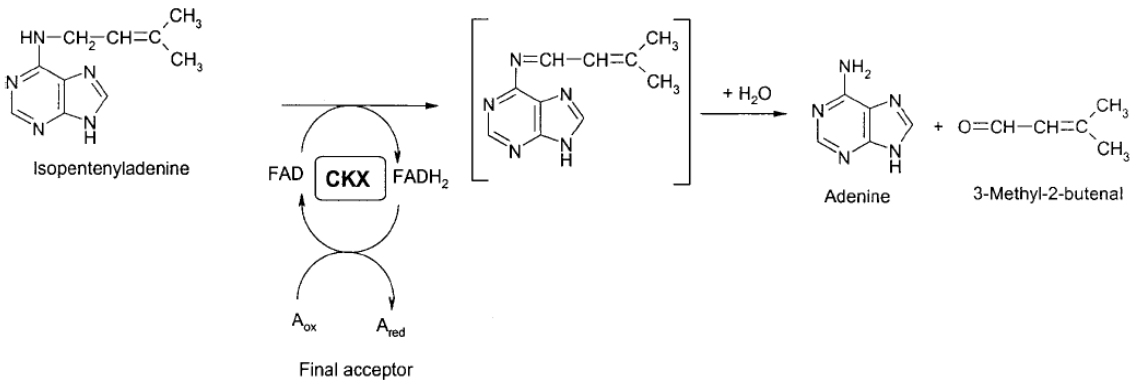


Figure 7: Scheme of isoprenoid CK degradation by CKX (Werner *et al.*, 2006). CKX - cytokinin dehydrogenase; FAD – flavin adenine dinucleotide.

The enzyme consists of two domains – GHS and substrate binding motif (Schmulling *et al.*, 2003). GHS is a FAD (flavin adenine dinucleotide) binding domain, where covalently bound FAD cofactor facilitates redox catalysis (Frebort *et al.*, 2011).

The occurrence of both glycosylated and non-glycosylated isoforms of CKX was reported in plants, with higher activity of glycosylated enzyme (Motyka *et al.*, 2003; Motyka *et al.*, 1996; Kaminek and Armstrong, 1990). Substrate specificity of CKX isoforms varies for distinct CKs types as well as their expression patterns (Werner *et al.*, 2006). Some CKX isoforms are able to degrade *cis*Z e.g. ZmCKX1 from maize and apoplastic AtCKX5 and AtCKX6, vacuolar AtCKX1 and cytosolic AtCKX7 from *Arabidopsis* (Gajdosova *et al.*, 2011). Latest reports brought also evidence that some CKX enzymes are able to degrade not only CK bases, nucleosides and nucleotides but also N9 glucosides (Šmehilová *et al.*, 2009).

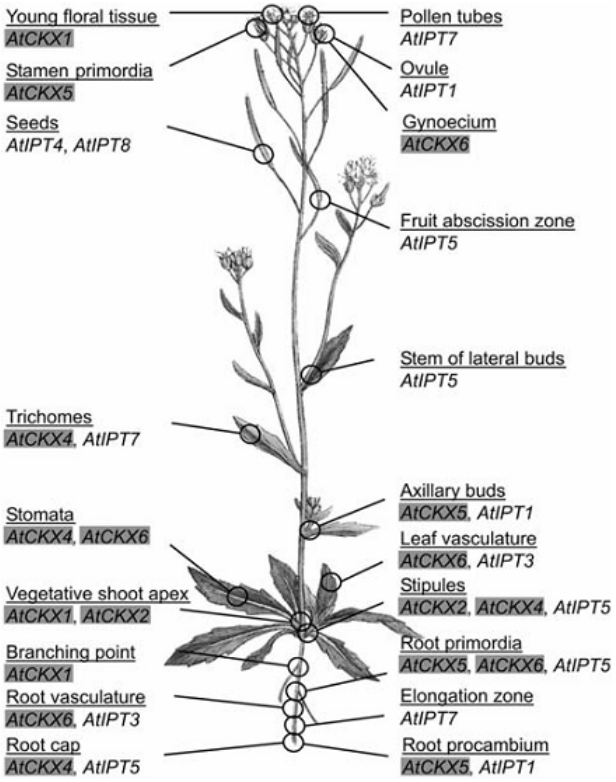


Figure 8: Expression patterns of CKX and IPT genes. Data are based on promoter-GUS fusion genes (Werner *et al.*, 2006).

3.1.6 CK perception and signalling

3.1.6.1 Cytokinin signalling is a multistep signal transduction system

CK signaling represents two-component machinery, which ensures signal perception and its further transfer of information in the form of phosphorelay on effector protein (Figure 9). This

type of signaling is common for bacteria, yeast, slime mold, fungi and from eukaryots uniquely for plants (Maxwell and Kieber, 2004). Plants possess an additonal component and its transduction system is named as multistep signal transduction system. The cue is sensed by hybrid histidine kinase in plants, usually dimer, which autophosphorylates itself at conserved histidine residue, phosphoryl group is then transferred to the aspartate at receiver domain. The phosphorelay continues via histidine of histidine phosphotransfer protein and finally is transmitted to the aspartate of receiver domain of response regulator, which mediates output (Maxwell and Kieber, 2004; Lohrmann and Harter, 2002).

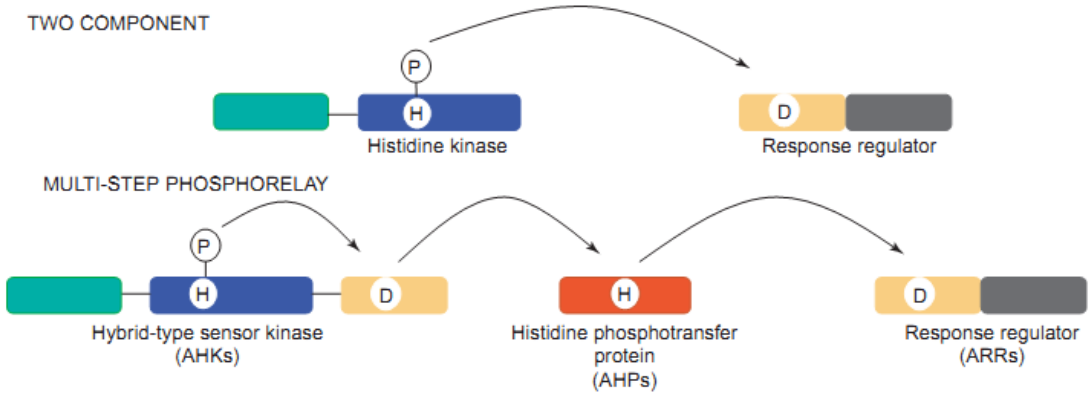


Figure 9: Schemes of two-component and multistep signal transduction system. (Ferreira and Kieber, 2005). P - phospate, H- histidine, D – aspartate.

3.1.6.2 CK receptors

3.1.6.2.1 Discovery of CK receptor

Initial discovery of a putative CK receptor started the elucidation of CK perception and signaling. An original assumption that CKI1, cytokinin independent 1, may represent CK receptor was not proved (Kakimoto, 1996). The presumption that CK receptor is a histidine kinase was confirmed after identification of the first *Arabidopsis* CK receptor CRE1, CYTOKININ RESPONSE 1 (Inoue *et al.*, 2001). Alleles of CRE1 were reported even earlier. One of them WOL (WOODEN LEG) was found to be related to the root vascular morphogenesis and expected to influence cell proliferation (Mahonen *et al.*, 2000). Another allele AHK4, ARABIDOPSIS HISTIDINE KINASE 4, and further homologs AHK2 and AHK3 were screened in search for amino acid sequence similarity of transmiter domains of CKI1 (Ueguchi *et al.*, 2001a).

3.1.6.2.2 Structure of *Arabidopsis* CK receptors

In *Arabidopsis*, three CK recepors – AHK2, AHK3 and AHK4 (CRE1) – have been identified. CK receptor represents a hybrid histidine kinase (Figure 10). The hybrid histidine kinase consists of C-terminal histidine kinase fused with receiver domain of response regulator at C-terminus (Suzuki *et al.*, 2001). Primary structures of *Arabidopsis* CK receptors are rather similar (Ueguchi *et al.*, 2001a).

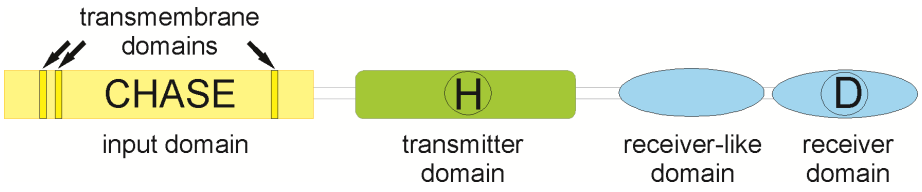


Figure 10: Structure of CK receptor. H - histidine, D – aspartate.

AHK4 contains two and AHK2 with AHK3 three putative transmembrane sequences at the C-terminus (Ueguchi *et al.*, 2001a). The cytokinin-binding cyclases/histidine kinases associated sensory extracellular, CHASE, domain is present between the transmembrane segments (Mougel and Zhulin, 2001; Anantharaman and Aravind, 2001). The CHASE domain is site for CK binding (Anantharaman and Aravind, 2001). Following transmitter domain possesses histidine kinase activity and encompasses conserved histidine residue that can be autophosphorylated. N-terminal part of the protein consists of receiver and receiver-like domain. Their sequences are similar but receiver-like domain has substituted conserved aspartate with glutamate in AHK3 and AHK4 therefore may not be relevant for phosphorelay (Ueguchi *et al.*, 2001a).

CK receptors are membrane localized proteins as it was predicted after their sequence analysis. The membrane localization was supported by observation that AHK4 expressed in fission yeast fed with ^{3}H iP retained all radioactivity in membrane fraction (Yamada *et al.*, 2001) and AHK3-GFP fusion protein which associated with plasma membrane (Kim *et al.*, 2006). Uniquely among AHKs only CRE1 shows phosphatase activity that is exhibited in absence of CK (Mahonen *et al.*, 2006).

3.1.6.2.3 *cis*Z is recognized by CK receptors

HKs respond to CKs differentially. In *Arabidopsis,* affinity to *cis*Z was observed only for AHK3 and it was significantly lower than to iP or *trans*Z (Romanov *et al.*, 2006). CRE1 does not respond to *cis*Z (Inoue *et al.*, 2001; Spíchal *et al.*, 2004), whereas AHK3 mediates CK response elicited by high concentrations of *cis*Z (Spíchal *et al.*, 2004).

All identified cytokinin receptors of maize were able to respond to *cis*Z (Yonekura-Sakakibara *et al.*, 2004).

3.1.6.3 Arabidopsis histidine phosphotransfer proteins (AHPs)

AHPs are proteins shuttling phosphoryl group from the receiver domain of HK to the receiver domain of response regulator (Imamura *et al.*, 1999). *Arabidopsis* contains 6 AHPs (Miyata *et al.*, 1998; Suzuki *et al.*, 1998; Suzuki *et al.*, 2000). Amino acid sequences of AHPs are very similar and each of AHPs consists of only His phosphotransfer domain with crucial histidine residue that serves as phosphorylation site. AHP6 is the only AHP that does not possess the conserved residue and thus lacks ability of phosphorelay. Therefore it is also designated as pseudo AHP - APHP1 (Suzuki *et al.*, 2000). AHPs are expressed differentially. AHP1 was abundantly found especially in roots but its expression was present also in leaves. Transcripts of AHP2, AHP3 and AHP5 were detected ubiquitously (Miyata *et al.*, 1998; Suzuki *et al.*, 1998; Suzuki *et al.*, 2000). AHP4 expression was almost undetectable in plants and low levels of AHP6 occurred in roots (Suzuki *et al.*, 2000). Cellular localization of AHP2-GFP was largely confined to cytoplasm (Imamura *et al.*, 2001). However, contradictory data were reported concerning behaviour of AHPs after CK treatment. Some authors observed migration to the nucleus (Yamada *et al.*, 2004) while others did not (Punwani *et al.*, 2010).

3.1.6.4 Arabidopsis response regulators (ARRs)

Last step of CK signaling in *Arabidopsis* is exerted by ARRs (Imamura *et al.*, 1998). According to the structure they can be divided into two groups - type A ARRs (D'Agostino *et al.*, 2000; Hwang *et al.*, 2002) and type B ARRs (ARR1-2, ARR10-14, ARR18-21, ARR23) (Tajima *et al.*, 2004).

A-type ARRs were identified as primary upregulated genes after CK treatment (D'Agostino *et al.*, 2000). Similarly to a bacterial response regulator CheY they lack a typical output domain (D'Agostino and Kieber, 1999). A-type ARRs consist of only receiver domain with short C and N- terminal extensions (Imamura *et al.*, 1998). The receiver harbors conserved D_2-D_1-K (Asp-Asp-Lys) residues, where D_1 is site for phosphorylation (Imamura *et al.*, 1999). Phosphoaccepting nature of ARR was demonstrated for ARR3, ARR4 and ARR6 (Imamura *et al.*, 1998). ARR proteins can be localized in nucleus (ARR7, ARR15), cytoplasm (ARR16) or in both compartments (ARR4) (Kiba *et al.*, 2002; Sweere *et al.*, 2001). ARR3-6,8,9 negatively regulate CK signaling and have partially overlapping functions (To *et al.*, 2004;

Lee *et al.*, 2007). ARR4 modulates red light signaling via interaction with PhyB (Sweere *et al.*, 2001) and expression of ARR9 is regulated by circadian clock (Ishida *et al.*, 2008).

ARRs of B-type are transcriptional regulators. They contain receiver domain and additional GARP motif (Tajima *et al.*, 2004). The receiver domain contains phosphoaccepting site and the GARP motif is necessary for DNA binding (Tajima *et al.*, 2004). The sole exception is ARR23, whose GARP domain is truncated (Hwang *et al.*, 2002). Expression patterns of B-type ARRs are distinctive as it is depicted in Figure 11.

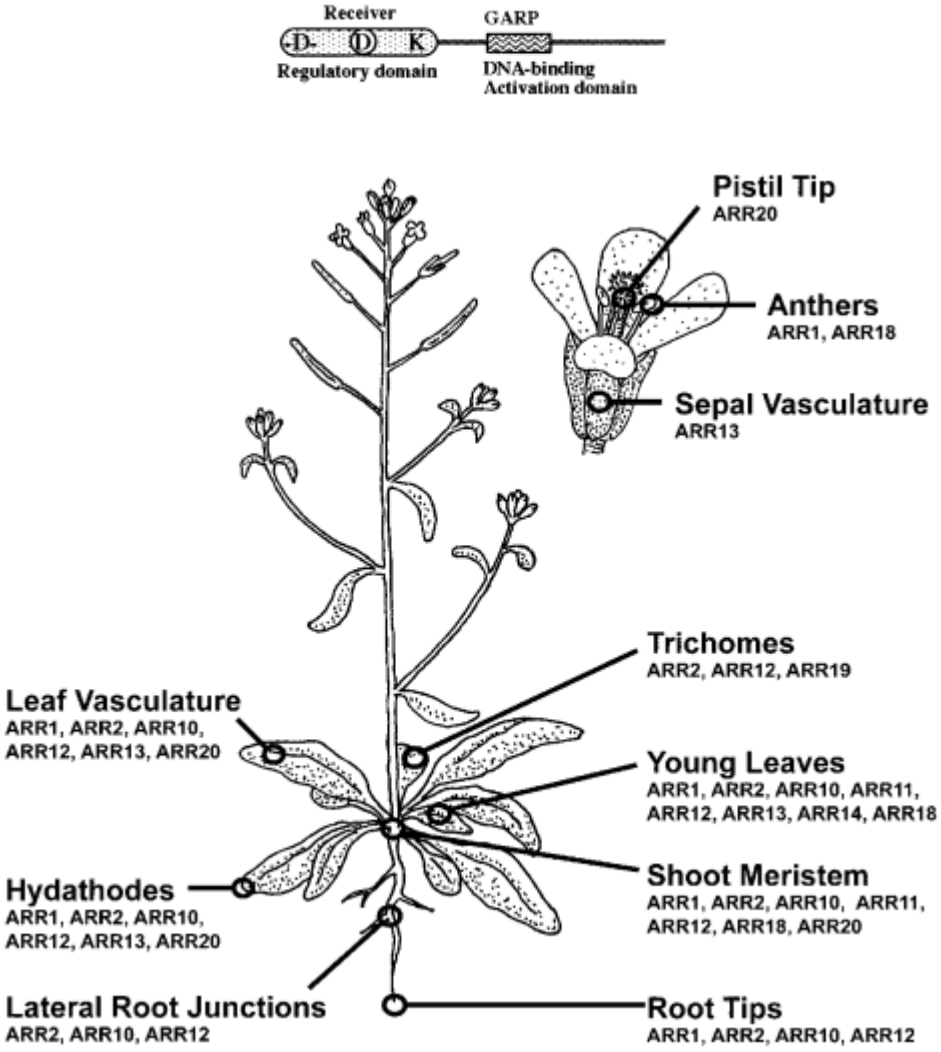


Figure 11: Structure and expression pattern of B-type ARRs (Tajima *et al.*, 2004; Mason *et al.*, 2004).

3.1.7 Mutants in CK biosynthesis and signalling

Mutants provide good basis for identification of gene and its product function. Sometimes they supply more information but usually they bring out more questions as it it for *cis*Z function.

3.1.7.1 Biosynthetic mutants related to cis-zeatin

3.1.7.1.1 Bacterial monooxygenase *miaE* mutant

Low CK activity has caused limited interest in *cis*Z since its discovery, but few hints pointing to potential physiological significance of free *cis*Z were published. Most of them describe increased *cis*Z levels under growth limiting conditions (Dobra *et al.*, 2010; Pertry *et al.*, 2009; Blagoeva *et al.*, 2003) but the strongest evidence was provided by bacterial *miaE* mutant lacking the hydroxylation step leading to *cis*Z formation. Thus the mutant is 2-methylthio *cis*-zeatin riboside (MeS-*cis*ZR) deficient but presence of prenylated adenine in tRNA is preserved (Persson and Bjork, 1993). Modification of tRNA including prenylation and hydroxylation improves speed and fidelity of proteosynthesis (Urbonavicius *et al.*, 2001). Hence *miaE* was not influenced by proteosynthesis-related defects from missing prenylation and allowed to characterize exclusively an impact of missing hydroxylation on the bacteria. Aerobically grown *miaE* mutant could not utilize intermediates from citric acid cycle (fumarate, malate and succinate while acetate and citrate could have been utilized with some problems) and did not excrete iron-chelating siderophores (Bjork *et al.*, 1999). Addition of siderophores to the media rescued *miaE* to resume the growth. The authors concluded that hydroxyl group of *cis*Z plausibly indicates the availability of oxygen in the environment and may influence the central metabolism. Several modes of action explaining observed phenotype were implied

- absence of prenyl iP hydroxylation in tRNA (*cis*Z deficiency) caused defects in proteosynthesis;
- prenyl hydroxylated tRNA could function in some other, potentially enzymatic, reaction than proteosynthesis;
- some other compounds than tRNA, e.g. enzymes of citric acid cycle, might have been hydroxylated by tRNA IPT, which may alter their function;
- missing regulator of central metabolism.

3.1.7.1.2 Arabidopsis atipt2, atipt9 and atipt2 9 mutants

Single T-DNA insertional mutants of *Arabidopsis* tRNA IPTs, AtIPT2 and AtIPT9, contained lowered levels of *cis*Z and its derivatives. Any mutant phenotype was not identified for *atipt2, a*lthough levels of free and tRNA-bound *cis*Z-type CKs in *atipt2* were lower than those in *atipt9 (Miyawaki et al., 2006).* On the contrary, *atipt9* and *atipt2 9* double mutant suffered from chlorosis (Miyawaki *et al.*, 2006) and growth of *atipt2 9* was severely retarded

(Vadassery *et al.*, 2008). Levels of endogenous *cis*Z CKs were reduced both in *atipt2* and *atipt9* (*atipt2*>*atipt9*) and *atipt2 9* lacked *cis*Z CKs completely. Analysis of tRNA-bound *cis*ZR showed the major impact of *atipt2* on *cis*ZR content as its abundance was decreased in *atip9*>*atipt2* and under detection limits for *atipt2 9*). Mutation in *atipt9* resulted in severe reduction of iPR in tRNA suggesting that observed phenotypes might have resulted rather from impaired tRNA prenylation and related inefficient proteosynthesis than *cis*Z itself (Miyawaki *et al.*, 2006; Urbonavicius *et al.*, 2001). Both tRNA IPT genes were expressed ubiquitously with prominent expression patterns in proliferating tissues, however AtIPT9 transcripts seemed to be more abundant than those of AtIPT2 (Miyawaki *et al.*, 2004).

3.1.7.1.3 Overexpression of human tRNA-IPT in *Nicotiana tabacum*

Constitutive overexpression of human tRNA-IPT under CaMV 35S promoter in tobacco resulted in numerous phenotypic alterations resembling weak increase in CK. Plants exhibited decreased apical dominance, reduced height, thicker and rigid stems and greener leaves of rounder shape and containing more chlorophyll compared to wild-type. Flowers were malformed, pollen production was defective resulting in male sterility and plants produced seeds with low viability (Golovko *et al.*, 2007). Similarly to *atipt* mutants, the highest differences between various transformed tobacco lines were not found in *cis*Z but in iP derivatives abundance.

3.1.7.2 Mutants in CK receptors

None of the single CK receptor mutant seedlings showed significant phenotype (Ueguchi *et al.*, 2001b; Nishimura *et al.*, 2004). However, greening of *ahk3-1* calli could be obtained only with high doses of *trans*Z suggesting reduced CK sensitivity. Mutant *ahk4-1* exhibited even more apparent cytokinin-insensitivity (Nishimura *et al.*, 2004). Double mutant combinations caused shoot growth retardation only in *ahk2-1 ahk3-1*, which also had impaired ability to respond to exogenous CKs as the chlorophyll degradation could not be rescued by application of CK. Triple *ahk2-1 ahk3-1 ahk4-1* mutant seedlings were dwarfed with narrow primary roots, reduced root meristematic region and enhanced formation of adventitious roots. Decreased number of leaf primordia was a result of reduced cell number and thus of smaller size of shoot apical meristem (Nishimura *et al.*, 2004). Leaves of the triple mutant were small due to low division rate, subsequent smaller number of cells per organ (Nishimura *et al.*, 2004) and also smaller size of leaf cells (Riefler *et al.*, 2006). Flowering of the mutant was delayed and flowers sterile as the anthers did not dehiscence and contained very limited

amount of viable pollen (Nishimura *et al.*, 2004). As expression of CK receptors occurred in overlapping domains in almost whole plants, any conclusion about specific function could not be drawn (Nishimura *et al.*, 2004). Phenotypic analysis of additional double mutant alleles *ahk2-5 ahk3-7* revealed faster germination in white, red and far red light and darkness additionally mutants formed also lateral roots of second order in contrast to wild-type (Riefler *et al.*, 2006). Based on these experiments it is obvious that AHK4 is involved mainly in CK-related processes associated with roots, AHK3 influences shoot related events and AHK2 pronounces the effects of AHK3 and AHK4 (Riefler *et al.*, 2006).

3.1.7.3 AHP mutants

Single AHP mutants were indistinguishable from wild-type plants, some of double mutants were slightly CK insensitive and the insensitivity increased in higher order mutants. AHP1,2,3,5 confer to the positive regulation of CK signaling and solely AHP4 may lead to its slight repression (Hutchison *et al.*, 2006).

3.1.7.4 ARR mutants

3.1.7.4.1 A-type ARRs mutants

Among single mutants of ARRs just *arr4* and *arr5* exhibited subtle rosette phenotypes and mutant plants showed enhanced sensitivity to exogenous CK with increasing number of disrupted ARRs (To *et al.*, 2004).

3.1.7.4.2 B-type ARRs mutants and overexpressing lines

Triple insertional mutant arr1-3 arr10-5 arr12-1, which are expressed almost ubiqutously in wild-type (Tajima *et al.*, 2004), exhibited CK deficient traits – reduced growth, larger seeds, reduced response to CK treatment (Argyros *et al.*, 2008). Overexpressed ARR1 caused disordered proliferation (Sakai *et al.*, 2001) Overexpression of ARR14 caused formation of adventitious shoots. ARR20 and ARR21 were predominantly expressed in reproductive organs. Whereas overexpression of ARR20 caused sterility, ARR21 led to formation of callus-like proliferations and induced expression of auxin repressors (Tajima *et al.*, 2004).

4 Material and methods

4.1 Chemicals

All cytokinins were obtained from Olchemim Ltd. (Olomouc, Czech Republic). 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). The deuterium-labelled CK standards and inhibitor of ethylene biosynthesis L-α-[(2-aminoethoxy)vinyl]glycine (AVG) were purchased from Olchemim Ltd. (Olomouc, Czech Republic). Auxin analog α-naphtalene acetic acid (NAA) were bought from Calbiochem (San Diego, California, USA) and sucrose from Lachema (Neratovice, Czech Republic). Other chemicals were purchased from Sigma-Aldrich Inc. (St.Louis, MO, USA).

4.2 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 July 2008 in Prague in the Czech Republic. Seeds of *Arabidopsis thaliana* Columbia ecotype 0 (Col-0) were used as a wild type. Seeds of T-DNA insertional mutants were obtained from The Nottingham Arabidopsis Stock Centre (*atipt2*), those of *atipt2 9* was generously provided from Prof. Tatsuo Kakimoto (Department of Biology, Osaka University, Osaka, Japan) and *atipt9* from Dr. Petr Tarkowski (Faculty of Science, Palacký University Olomouc, Czech Republic).

4.3 Chlorophyll retention bioassay

Oat (*Avena sativa* L. cv. Abel) seeds were soaked for 24 hours in aerated distilled water (26°C, 16h light /8h dark) and sown into saturated perlite with a two-fold concentrated Knop´s nutrient solution. Plants were cultivated in a growth chamber (SANYO MLR 350H; Sanyo, Osaka, Japan) at 18h light/6h dark photoperiod (photon flux of 19 995 lx), 20°C/18°C and ca. 80% relative humidity. The first fully developed leaves were excised from 10 d old plants when the second leaf just started to develop. The leaf apexes were cut to 7 cm long segments and incubated in test tubes containing 1 ml CK solutions for four days 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 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 (ca. 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 in 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.

4.4 Tobacco callus bioassay

CK-dependent tobacco (*Nicotiana tabacum* cv. Wisconsin 38) callus derived from a four-week old culture was cultivated on solid MS media (Murashige and Skoog, 1962) supplemented with sucrose (30 g l^{-1}), NAA (1 mg l^{-1}) and tested CK (in a concentration ranging from 0.64 nM to 10 μM). CK activities were expressed as average fresh weight of tissue per flask after seven weeks of cultivation in darkness at 23°C and compared in accordance with EC_{50} values (see above). Three independent experiments were set up for each CK. The stock calli were grown on the same media containing benzyladenine (0.2 mg l^{-1}). The control calli for the experiment were cultivated on media without supplied CKs. 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.

4.5 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]*transZ* (2.10^{-9} mol.l^{-1} each) uptake, accumulation and short-term metabolism were measured in CK-autonomous tobacco (Nagata *et al.*, 1992) cell suspension (0.5 mL aliquots) according to Delbarre *et al.* (1996) as described in Petrášek *et al.* (2003). The BY-2 cells were harvested immediately after the addition of CK into liquid media (time 0 min) and after 5 min intervals during incubation. The uptake and long-term 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 a 2.5 mL water solution containing 42 to 47 kBq radiolabeled *cis*Z and *trans*Z, respectively. The incubation proceeded under continuous light (photon flux of 19 995 lx) at 20°C for 2, 5, 8, 24, 48 and 96 h. CKs were extracted and purified from 14-16 primary leaf segments (ca. 1 g FW) per sample; for each time interval

two independently incubated samples were analysed. Radiolabeled CK metabolites were analyzed by HPLC coupled with an on-line radioactivity detector as described by Gaudinová *et al.* (2005) and identified by comparing their retention times with authentic standards.

4.6 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 was determined by *in vitro* radioisotope assays based on the conversion of [2-^{3}H]labeled 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 the 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 tissue FW (corresponding to 0.02 mg protein g^{-1} FW for BY-2 cells). After incubation (1 and 4 h for oat and tobacco, respectively) at 37^{o}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). The analogous reaction conditions and the identical assay mixture were used for CKX analysis in dry seeds having been optimalized for each species (Stirk *et al.*, submitted). For CKX analysis in zucchini cotyledons, the TAPS-NaOH buffer was substituted by MOPS-NaOH (100 mM, pH 7.0) and the enzyme preparations equivalent to 175 mg tissue FW (corresponding to 0.28-0.36 mg protein g^{-1} FW) were applied and incubated for 1 h at 37^{o}C (Ananieva *et al.*, 2008). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

4.7 Analysis of endogenous cytokinins

Endogenous CKs were extracted by methanol/formic acid/water (15/1/4, v/v/v) from plant tissues, homogenized in liquid nitrogen, and purified using dual-mode solid phase extraction method (Dobrev and Kaminek, 2002). Cytokinin ribotides were determined as corresponding ribosides following their dephosphorylation by alkaline phosphatase. Analysis of CKs was carried out using HPLC/MS system as described by Dobrev *et al.* (2002)(2002) consisting of HTS-Pal autosampler (CTC Analytics, Zwingen, Switzerland), quaternary HPLC pump Rheos 2000 (Flux Instruments, Basel, Switzerland) and a Finnigan MAT LCQ-MS^n ion trap mass spectrometer (Finnigan, San Jose, CA, USA) equipped with an electrospray interface.

Detection and quantification were performed using a Finnigan LCQ operated in the positive ion full-scan MS/MS mode using a multilevel calibration graph with [^{2}H]labeled cytokinins as internal standards. Detection limits of different cytokinins were between 0.5 and 1.0 pmol/sample. Results represent averages of analyses of three independent samples and of two HPLC MS/MS injections for each sample.

4.8 Cultivation of Arabidopsis thaliana seedlings in vitro

Medium used for *in vitro* cultivation of *Arabidopsis* seedlings contained half strength basal salt MS (Murashige and Skoog, 1962) containing 10 g.l^{-1} sucrose and 10 g.l^{-1} agarose (pH 5.7) and is referred as MS/2 in the text. Sterilized CKs (*cis*ZR and *trans*ZR) were added after autoclaving to achieve final concentration of 0.5 μM and NAA (0.1 μM) and, in some cases, aminoethyoxyvinyl glycine (AVG; 2 μM) were supplemented. *Arabidopsis* seeds were surface sterilized for 2 minutes with 70% ethanol, for 10 minutes with 1% solution of SAVO (Bochemie, Bohumin, Czech Republic) containing a drop of Tween 20 (Promega, Madison, USA) and rinsed with sterile distilled water. Afterwards, seeds were sown on square plates, stratified for 72 hours at 4°C in darkness and grown vertically for 7 days (experiments solely with wild-type Co-0 *Arabidopsis*) or 10 days (experiments with tRNA-IPT mutants) at 20°C under 18h/6h light/dark regime. Plants grown in soil were cultivated at 20°C/18°C, 18h/6h light/dark and 80% relative humidity (RH).

4.8.1 Chlorophyll measurements in Arabidopsis

Shoots and leaves of *in vitro* grown *Arabidopsis* seedlings were utilized for chlorophyll content measurements. Chlorophyll was extracted with 80% ethanol according to Kaminek *et al.* (1987), optical absorbance was measured at Helios Beta spectrophotometer at 643.5 nm and 661 nm. Content of chlorophyll a and chlorophyll b was calculated according to Sramek and Dubsky (2011).

4.8.2 Root length and seed measurements in Arabidopsis

Roots of vertically *in vitro* grown *Arabidopsis* seedlings and seeds were observed under stereo microscope Nikon SMZ1500 and photographed. Photographs were used for image analysis with image analysis software NIS Elements AR 3.0 and various parameters such as length of primary root, seed width and length were measured.

4.8.3 GUS-staining

Five days old wild type *Arabidopsis thaliana* were infiltrated with staining solution and incubated for 30 minutes in 37ºC. Staining solution contained 100mM Na_2HPO_4, 50mM $K_3Fe(CN)_6$, 50mM $K_4Fe(CN)_6.H_2O$, 1mg.ml^{-1} 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt (X-Gluc). Stained seedlings were afterwards transferred to 70% ethanol and observed under microscope Nikon Eclipse E600. Bright field images of at least 14 replicates were inspected.

4.9 Presentation of the results

If not otherwise stated, all analyses were repeated two to four times. Statistical variations of the results are expressed as the mean ± standard deviation in the figures as well as in the tables presented in Results.

5 Results

5.1 Occurrence of *cis*Z-type cytokinins in the plant kingdom

In order to determine the occurrence of *cis*Z-type CKs in plants and to depict their contribution to the whole CK contents, the CK profiles and concentrations were analysed in more than 20 plant species by HPLC-MS/MS. For that purpose, the CK spectra were divided into two groups of interest including (a) *cis*Z-type CKs and (b) non-*cis*Z-type CKs (represented by *trans*Z, iP and DHZ and their derivatives). In accordance with the structures and physiological activities, both *cis*Z- and non-*cis*Z-type CKs were classified into four functionally different groups, comprised as shown in Figure 12. The total amount of CKs among all tested species varied between ca. 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 more than 50% of the whole cytokinin content (Figure 12). Especially 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 major cytokinins (Figure 12). The most abundant CK metabolites detected in these genera were *cis*Z-O-glucoside (*cis*ZOG) and its riboside (*cis*ZROG), representing altogether more than 90% of total CKs. In dicot plants, for instance, a similar CK profile with a clear predominance of *cis*ZOG and *cis*ZROG was found in *Manihot sp.* (Euphorbiaceae) leaves (Figure 12). Relatively high levels of *cis*Z-type CKs (exceeding 65% of the total CKs) were also detected in leaves of *Nicotiana tabacum*, however, with *cis*Z-N7-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 with no apparent parallel with evolutionary komplexity.

5.2 Activity of *cis*Z derivatives in cytokinin bioassays

Most physiological activity of Z has been attributed for years to *trans*Z, while *cis*Z has been considered mainly as a non-active or only weakly active CK. For this reason we compared the biological activity of *cis*Z and *trans*Z and their derivatives (ribosides, O- and N9-glucosides)

in classical CK bioassays based on (*a*) retention of chlorophyll in excised oat and wheat leaves and (*b*) stimulation of cell division.

5.2.1 Chlorophyll retention bioassays

Darkness-induced senescence is associated with plastid deterioration and increased chlorophyll degradation. CKs are known to delay darkness-induced senescence (Richmond and Lang, 1957). For this reason, biological activities of exogenously applied derivatives of *cis*Z and their *trans*Z counterparts were tested in chlorophyll retention bioassays. Two representatives of *Poaceae* family, oat and wheat, were selected for the assays because both species were found to contain prevalent amounts of *cis*Z derivatives (Sykorova *et al.*, 2008).

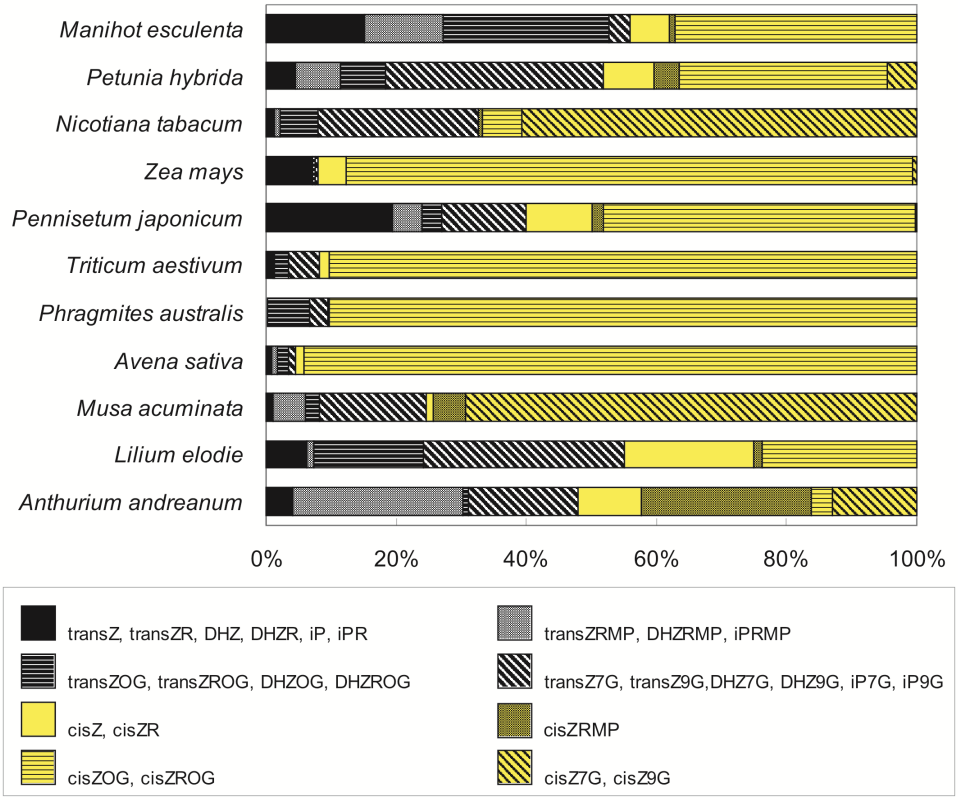


Figure 12: Percentual proportion of isoprenoid CKs groups in leaves of several plant species and contribution of *cis*Z-type CKs to the whole CK pool. Plant species are arranged from the oldest to the youngest in evolutionary terms (from the bottom to the top); the CK derivatives are divided into 8 groups of interest according to their structure and activity. Yellow coloured bars represent *cis*Z-type CKs and black and white bars show non-*cis*Z-type CKs (derivatives of iP, DHZ, *trans*Z).

5.2.2 Oat leaf chlorophyll retention bioassay

In the oat leaf senescence bioassay all tested *trans*Z-type CKs suppressed chlorophyll degradation efficiently (Fig.2 - skutečné číslo obrázku). The highest response was found for *trans*Z-O-glucoside (*trans*ZOG) exhibiting biological activity already at concentration 4 μM, while *trans*Z, and its riboside (*trans*ZR) were active at ≤ 20 μM. Surprisingly, high ability to

delay chlorophyll degradation was found for *trans*Z-N9-glucoside (*trans*Z9G). All *cis*Z-type CKs were also effective in delaying dark-induced senescence but with rather lower efficiency (between 5- to 50-times when compared on the basis of EC_{50} values) than their *trans* counterparts (Figure 13). The differences among *cis*Z derivatives were more pronounced. The highest chlorophyll retention was observed after 500 μM *cis*Z*O*G and *cis*ZR application, when ca. 70 to 92 % of the maximum responses induced by corresponding *trans* isomers were reached. The lowest detectable concentrations required for positive effects on chlorophyll retention were 100 μM for *cis*Z and *cis*ZR and 500 μM for *cis*Z*O*G. The lowest activity was recorded after treatment with *cis*Z-N9-glucoside (Figure 13).

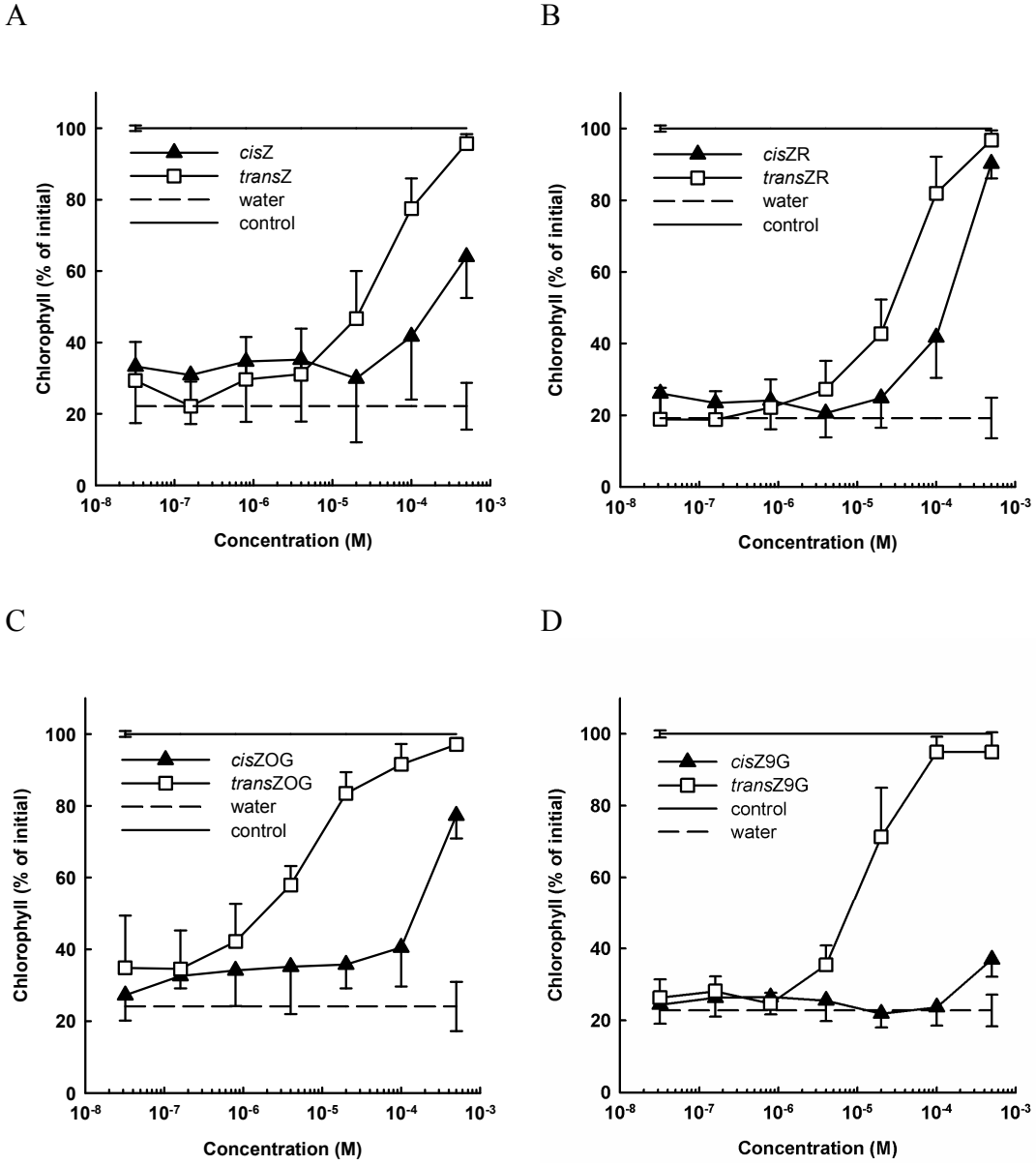


Figure 13: Ability of Z isomers and their derivatives to retard dark-induced senescence in *Avena sativa*. Free bases (A), ribosides (B), O-glucosides (C), N9-glucosides (D). Control value represents level of chlorophyll at the beginning of the experiment, water – indicates chlorophyll content in leaf segments after 4 days long incubation in darkness supplied only with water. Error bars represents standard deviation.

To check the chlorophyll retention data, inhibition of plastid disintegration in oat leaves treated with *cis*- or *trans*Z in leaf specimens was confirmed by images from laser scanning microscope (Figure 14).

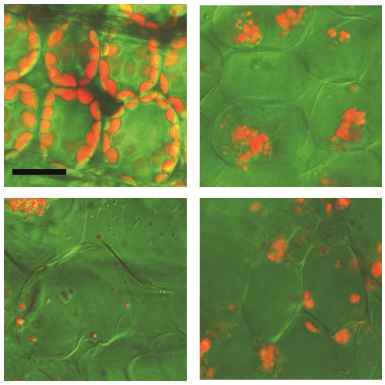
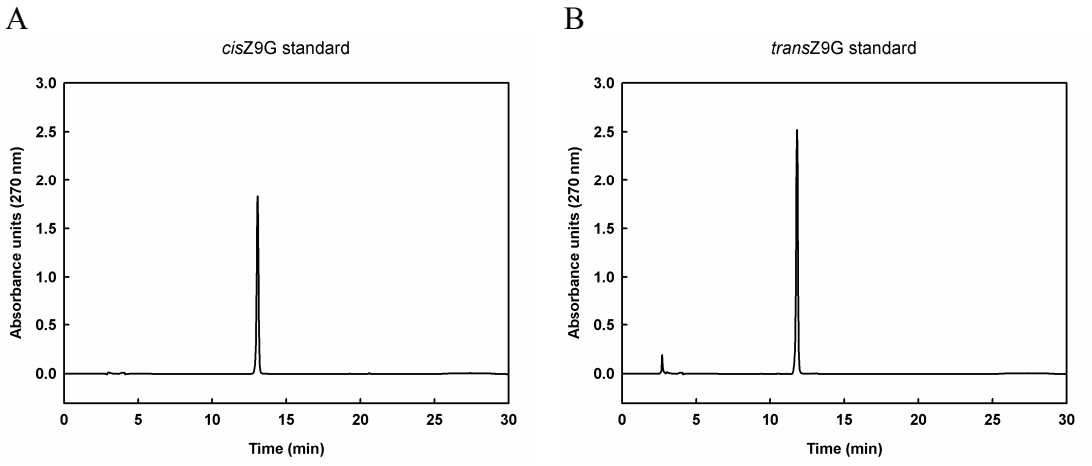


Figure 14: Images of oat leaf mesophyll cells and their chloroplasts (original red autofluorescence). From the upper left corner to the right – Control (fresh oat leaves before experiment), leaves treated with *cis*Z, water, *trans*Z after 10 days of cultivation in the dark. The bar represents 20μm.

5.2.2.1 Assesment of possible decay of cis- and trans-zeatin-N9glucosides during chlorophyll retention bioassay

Cytokinin N9-glucosides were repeatedly reported to be final deactivation products of CKs and presumed to possess none physiological activity. As *cis*Z9G (in lesser extent) and especially *trans*Z9G exhibited ability to delay chlorophyll degradation in oat leaf segments, we tested whether these derivatives were not subjected to a possible hydrolysis during the bioassay and whether their activity could not be attributed to a formation of free bases (*cis*Z, *trans*Z). Hence, possible breakdown of both N9-glucosides was analyzed in incubation solutions. However, no peaks that would indicate the presence of *cis*Z or *trans*Z (and/or other metabolites) were detected (Figure 15).



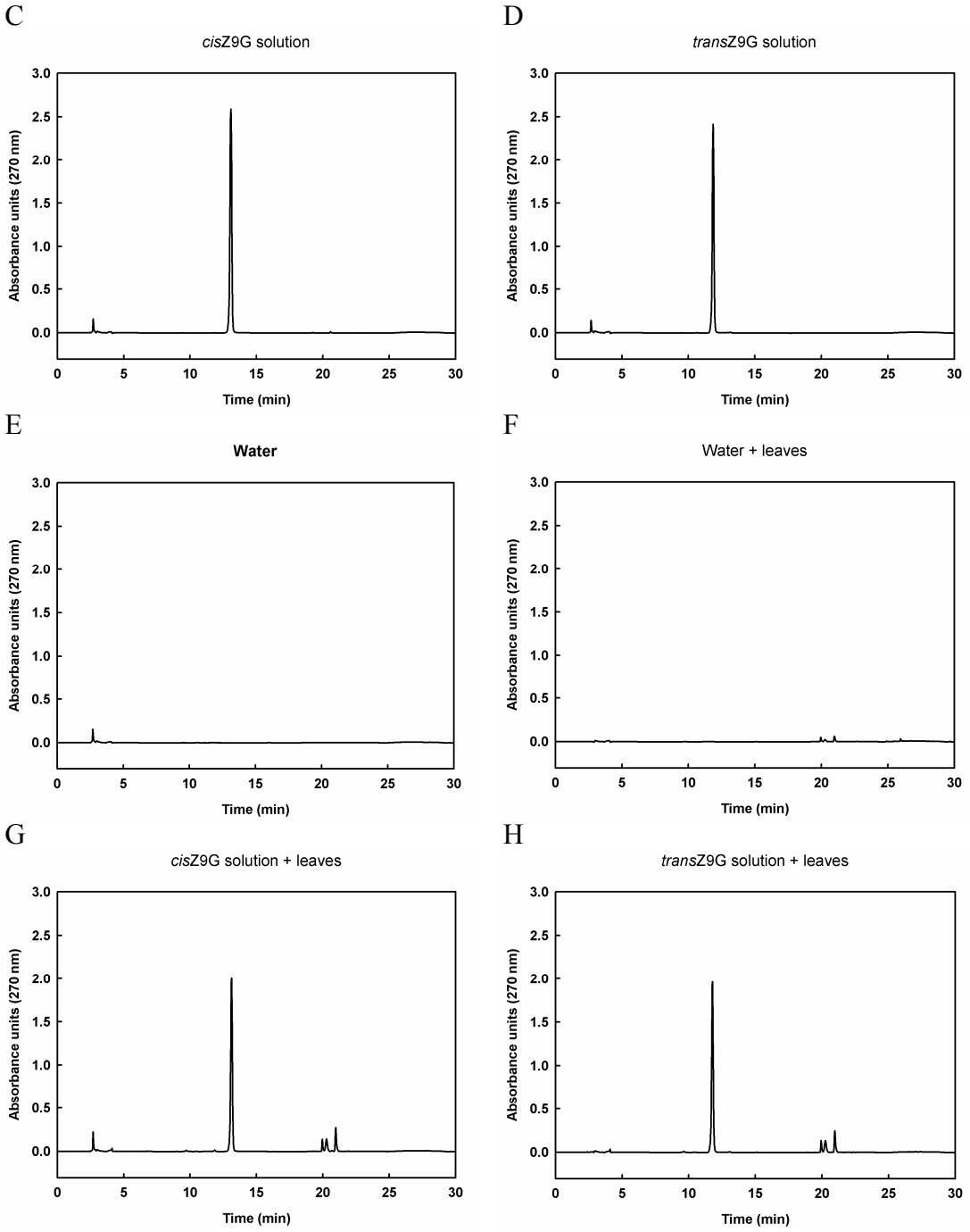


Figure 15: Chromatograms depicting potential decay of zeatin–N9-glucosides. HPLC chromatograms of the CK solutions (*cis*Z9G, *trans*Z9G standards) before experiment (A, B); the CK solutions (*cis*Z9G, *trans*Z9G) and water without leaf segments at the end of experiment (C, D, E); water and the CK solutions (*cis*Z9G, *trans*Z9G) at the end of experiment after incubation with leaf segments (F, G, H).

5.2.3 Wheat chlorophyll retention bioassay

Corresponding results demonstrating activities of *cis*Z-type CKs and their *trans* counterparts (free bases, ribosides) to delay dark-induced chlorophyll degradation were also obtained with excised wheat leaves in the same assay (Figure 16). Derivatives of *cis*Z derivatives showed comparable effects on chlorophyll retention in wheat as in oat leaf segments. Although in wheat none of them was able to prevent chlorophyll degradation as efficiently as in oat

segments. For both *cis*- and *trans*-isomer types, ribosides were again more active than free bases. Ability to hinder chlorophyll degradation was very weak for free *cis*Z (Figure 16). Similarly, the data indicating *cis*Z and *cis*ZR activities were obtained with maize leaf segments in a drop bioassay (data not shown).

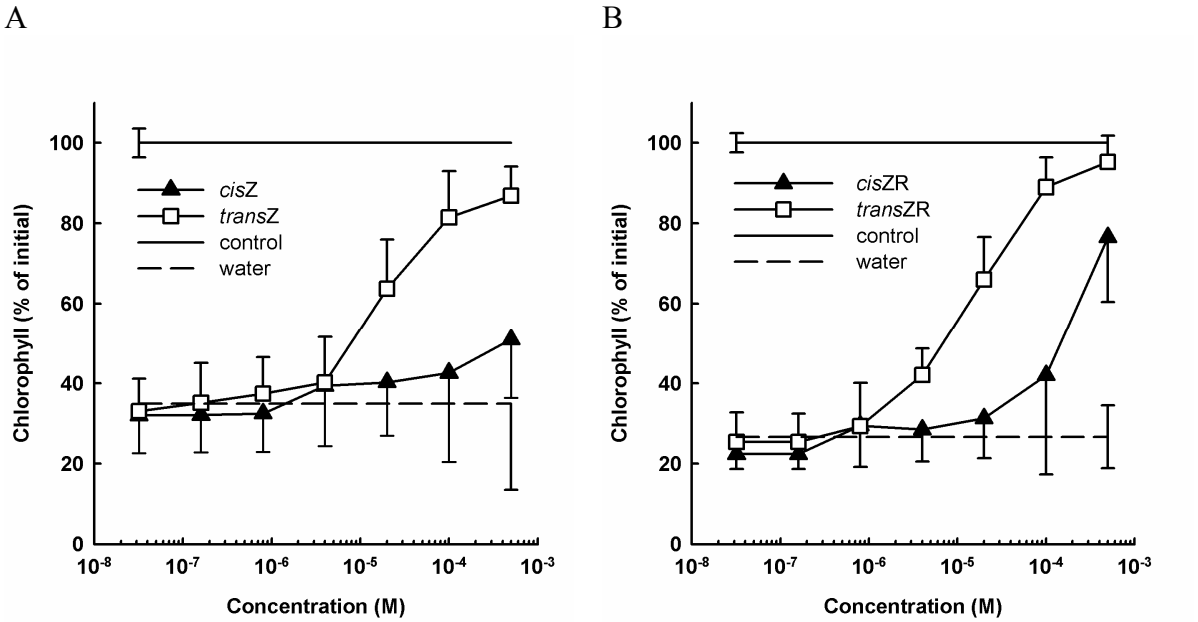


Figure 16: Effects of Z isomers on chlorophyll retention in *Triticum aestivum*. Free bases (A) and ribosides (B). Control value represents level of chlorophyll at the beginning of the experiment, water – indicates chlorophyll content in leaf segments supplied only with water after 4 days long incubation in darkness. Error bars represents standard deviation.

5.2.4 Tobacco callus bioassay

The capability of *cis*Z- and *trans*Z-type CKs to induce and maintain cell division was investigated using CK-dependent tobacco callus cultures. Biological activities of *cis*Z, *cis*ZR, *cis*ZOG and their corresponding *trans* counterparts are shown in Figure 17. All of the tested *trans* isomers considerably enhanced tobacco cell division. The lowest effective concentrations were 3.2 nM for *trans*Z, 16 nM for *trans*ZR and 80 nM for *transZO*G. All tested *c*Z-type CKs were remarkably effective in promotion of tobacco callus growth displaying activities in the order *cis*ZR > *cis*Z > *cisZO*G (based on EC_{50} values;Figure 17). The most pronounced biological response on callus growth was recorded for *cis*ZR at 400 nM, which represented over 90% of the highest activity observed for the corresponding *trans* isomer. The maxima of cell division stimulatory effects of *cis*Z and *cisZO*G forms were shifted to 2 μM and 10 μM, respectively. In general, the *trans*Z : *cis*Z activity ratio of assayed CK derivatives varied between ca. 3 and 27 when compared on the basis of EC_{50} values (Figure 17). Apparently a possible interchangeability of *trans*Z- by *cis*Z-type CKs, at least in terms of maintaining cell division, can be inferred.

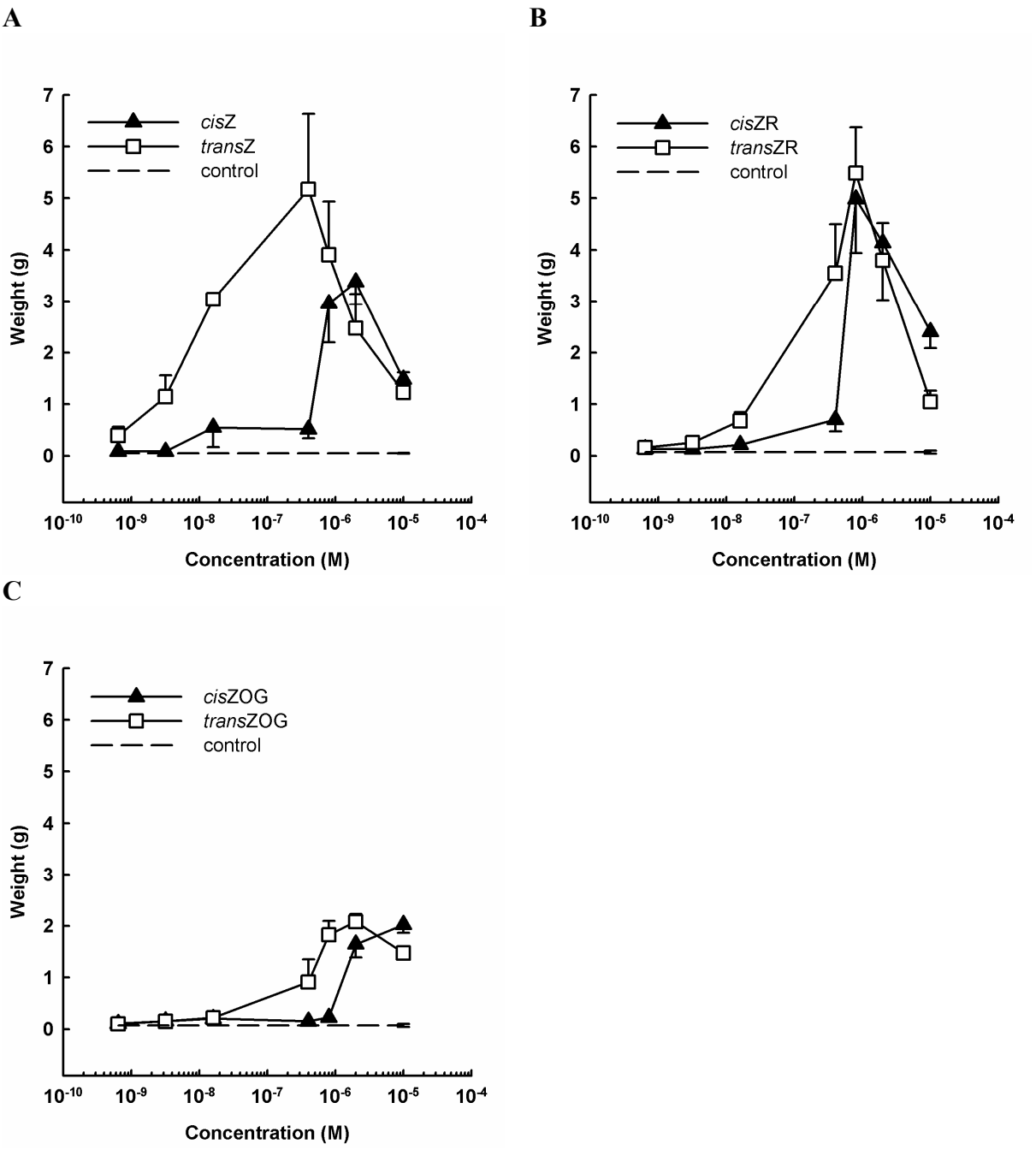


Figure 17: Ability of Z isomers to induce and maintain cell division in cytokinin-dependent tobacco callus. Free bases (A), ribosides (B), O-glucosides (C). Control value depicts the tissue cultivated on medium without supplied CK. Error bars represents standard deviation.

5.3 Metabolism of *cis*Z in plants

To obtain information concerning metabolism of *cis*Z and *trans*Z in plants, radiolabelled [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z were added either (*a*) to the detached oat leaves (long-term metabolism) or (*b*) into liquid media of tobacco BY-2 suspension culture.

5.3.1 Long-term metabolism of radiolabelled cis- and trans-zeatin in detached oat leaves

With respect to high activities of both *cis*- and *trans*Z-type CKs found in the oat chlorophyll retention bioassay it was decided to investigate the metabolism of radiolabelled [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z in detached oat leaves. The results of the long-term "feeding" experiments in oat leaf segments are shown in Figure 18. A majority of [^{3}H]*cis*Z was metabolized to adenine (up to 50 % within 8 h) and in lesser extent but progressively to adenosine (ca. 8 % after 96 h; Fig. 7A) indicating extensive degradation of *cis*Z by CKX. In accordance with the prevailing occurrence of endogenous *cis*ZOG in oat leaves (Figure 12), radiolabeled *cis*ZOG was detected as an abundant [^{3}H]*cis*Z metabolite (Figure 18). During the whole incubation, about 12 % of total metabolites was constantly retained in unmetabolised [^{3}H]*cis*Z form. The degradation products adenine and adenosine were also found as major metabolites of [2-^{3}H]*trans*Z in oat leaves (Figure 18). In contrast to *cis*Z, the application of [^{3}H]*trans*Z to oat leaves resulted in the formation of a greater number of CK forms including corresponding *N*7- and *N*9-glucosides (Figure 18). This suggested that the metabolic fate of both *cis*- and *trans*Z isomers differed in the type of glucosylation. Neither [^{3}H]*cis*Z nor [^{3}H]*trans*Z were found to be a subject of *cis*Z ↔ *trans*Z isomerization. Similarly, a distinct metabolism of [^{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).

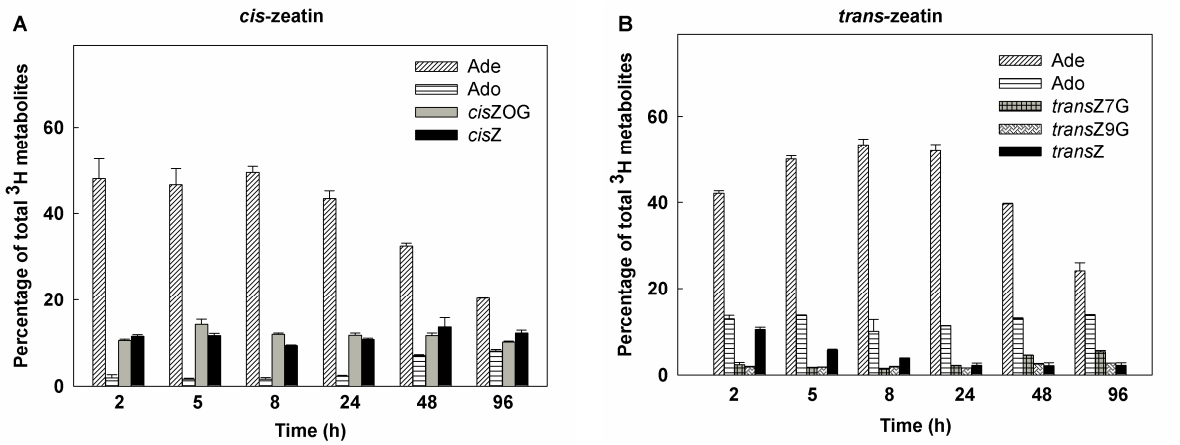


Figure 18: **Long-term metabolism of radiolabelled [^{3}H]*cis*Z (A) and [^{3}H]*trans*Z (B) in oat leaf segments.** Profiles of CK metabolites in corresponding time points (2, 5, 8, 24, 48, and 96 h after application) are shown. The values are expressed as a percentage of the total radioactivity taken up by the excised leaves. Error bars represent standard deviation. (Ade, adenine; Ado, adenosine; *cis*Z, *cis*-zeatin; *cis*ZOG, *cis*-zeatin-O-glucoside; *trans*Z, *trans*-zeatin; *trans*Z7G, *trans*-zeatin-N7-glucoside; *trans*Z9G, *trans*-zeatin-N9-glucoside).

5.3.2 Short-term metabolism of radiolabelled cis- and trans-zeatins in tobacco BY-2 cells

In order to acquire information about the metabolic fate of both *cis* and *trans* isomers of zeatin immediately after exogenous application of radiolabelled [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z,

tobacco BY-2 suspension culture was utilized. It was shown that both *cis*Z and *trans*Z were rapidly (within 15 min) and progressively accumulated in cultured tobacco cells with no preference for either of the isomers (data not shown). The fate of radiolabelled Z metabolites was determined in 4 time points during the first 15 min interval (0, 5, 10, and 15 min). Radiolabeled *cis*Z was metabolized in BY-2 cells to only few metabolites. 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 (from 8 % to 25 % of total counts) and in lesser extent with *cis*ZR (from 5 % to 6 %). Accumulation of adenine (from 4 % to 8 %) indicated degradation of *cis*Z by CKX (Figure 19). Aadenosine was detected only in minute amounts. Compared to *cis*Z, the application of [^{3}H]*trans*Z to BY-2 cells led to the detection of a greater number of corresponding metabolites including N- and O-glucosides. Similarly to *cis*Z, the prevailing metabolites of [^{3}H]*trans*Z were its corresponding ribotide (*trans*ZRMP; increase from 19 % to 39 % of total radioactivity), riboside (*trans*ZR; from 4 % to 6 %) and adenine (from 2 % to 3 %; Fig 6B). Also *trans*Z-*N*7- 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. Although the proportion of adenine and adenosine were lower than after *cis*Z application, the CKX catalysed degradation of *trans*Z had obviously a tendency to increase. Interestingly, any conversion of the labelled *cis* into the *trans* isomer and vice versa was not revealed in in the course of the experiments.

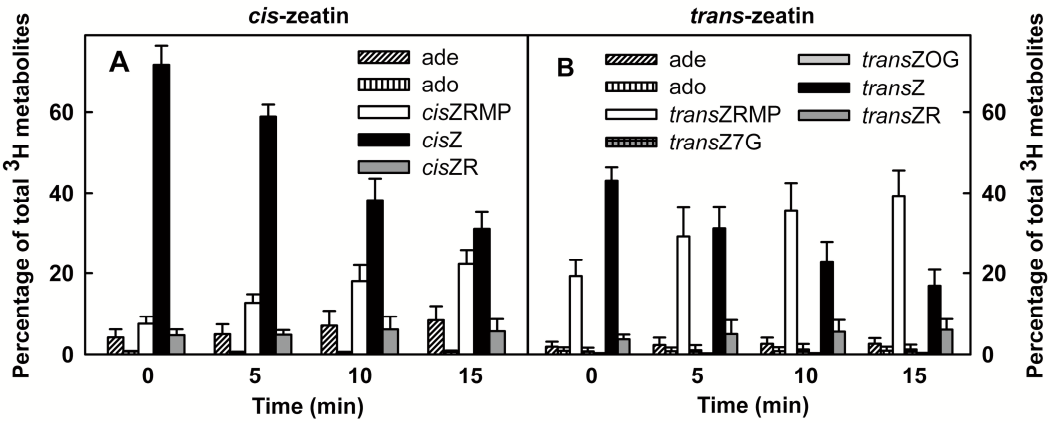


Figure 19: Short-term metabolism of radiolabelled [^{3}H]*cis*Z (A) and [^{3}H]*trans*Z (B) in tobacco BY-2 suspension culture. Profiles of CK metabolites in corresponding time points (0, 5, 10, and 15 min after application) are shown. The values are expressed as a percentage of the total radioactivity taken up by the cells and associated with particular CK metabolites Error bars represent standard deviation. (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). Samples were collected by Petr Klíma.

5.3.3 CKX activity and substrate specificity toward zeatin isoforms

With respect to large amounts of adenine and adenosine formed as products of *cis*Z and *trans*Z metabolism during oat long-term and BY-2 short-term feeding (Figure 19, Figure 18), degradation of [2-^{3}H]*cis*Z, [2-^{3}H]*trans*Z and [2-^{3}H]iP by CKX from crude extracts of the 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 demonstrates their higher or identical affinities for *cis*- than *trans*-isomer. iP was degraded at rates 1.6 to 4.8-fold and 5.3 to 11.7-fold higher than *cis*Z and *trans*Z, respectively (Table 1).

	iP	***trans*Z**	***cis*Z**
Oat leaves	0.134 ± 0.008	0.025 ± 0.003	0.028 ± 0.004
Tobacco leaves	0.268 ± 0.019	0.023 ± 0.002	0.100 ± 0.009
BY-2 cells	0.515 ± 0.025	0.081 ± 0.003	0.321 ± 0.040

Table 1 : Activity and substrate specificity of crude extracts of CKX enzymes from oat and tobacco leaves and BY-2 cells toward iP, *trans*Z, *cis*Z. The CKX activity was determined using [2-^{3}H]N^6-(2-isopentenyl)adenine (iP), [2-^{3}H]*trans*-zeatin (*trans*Z) and [2-^{3}H]*cis*-zeatin (*cis*Z), 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 is expressed in nmol adenine/mg protein.h.

Higher or identical affinity of CKX for *cis*Z compared to *trans*Z is not very common in plants. In general, *trans*Z and its riboside are usually better substrates for CKX than their *cis* counterparts (Armstrong, 1994). Having screened a great number of plant species at different developmental stages, higher affinity of CKX for *trans*Z compared to *cis*Z was found for most of them (data not shown). A part of these results have been included in two joint research papers (in which I was involved as a co-author) for selected plant materials such as zucchini cotyledons grown under different dark/light treatments (Ananieva *et al.*, 2004) and dry seeds of oats, maize and lucerne (Stirk *et al.*, submitted).

	iP	***trans*Z**	***cis*Z**
Control cotyledons, 10 days	0.573 ± 0.028	0.129 ± 0.009	0.062 ± 0.002
Both individually darkened cotyledons	0.306 ± 0.019	0.047 ± 0.004	0.029 ± 0.002
A single individually darkened cotyledon	0.627 ± 0.050	0.225 ± 0.015	0.081 ± 0.004
The other cotyledon which remained under light regime	0.782 ± 0.029	0.218 ± 0.013	0.109 ± 0.008

Table 2: Activity and substrate specificity of crude extracts of CKX enzymes from *Cucurbita pepo* (zucchini) cotyledons under different dark/light treatments toward iP, *trans*Z, *cis*Z. The CKX activity was

determined using [2-^{3}H]N^6-(2-isopentenyl)adenine (iP), [2-^{3}H]*trans*-zeatin (*trans*Z) and [2-^{3}H]*cis*-zeatin (*cis*Z), 2 μM each, as substrates. Enzyme activity was measured in 100 mM MOPS-NaOH buffer containing 75 μM 2,6-dichloroindophenol at pH 7.0 and is expressed in nmol adenine/mg protein.h.

	iP	***trans*Z**	***cis*Z**
Oat seeds	137.0 ± 23.8	21.5 ± 1.3	3.6 ± 0.0
Maize seeds	362.4 ± 26.0	13.0 ± 0.2	1.0 ± 0.1
Lucerne seeds	2.5 ± 0.1	0.6 ± 0.0	0.1 ± 0.0

Table 3: **Activity and substrate specificity of crude extracts of CKX enzymes from oat, maize and lucerne dry seeds toward iP, *trans*Z, *cis*Z.** The CKX activity was determined using [2-^{3}H]N^6-(2-isopentenyl)adenine (iP), [2-^{3}H]*trans*-zeatin (*trans*Z) and [2-^{3}H]*cis*-zeatin (*cis*Z), 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 in nmol adenine/mg protein.h.

5.4 Effects of exogenously applied *cis*- and *trans*-zeatin isomers on chlorophyll accumulation in *Arabidopsis thaliana*

CKs affect not only chlorophyll degradation but also facilitates its biosynthesis (Jelic and Bogdanovic, 1989). On the contrary, auxin reduces to some extent delay of formation of chlorophyll (Sunderland and Wells, 1968). In this experiment, *Arabidopsis thaliana* young seedlings were used to examine the impact of *cis*- and *trans*-zeatin isomers, applied either alone or in combination with auxin analog NAA, on chlorophyll accumulation. For this purpose, ribosides of *cis*Z and *trans*Z were chosen because of their high activities found in bioassays described previously.

The application of CK (0.5 μM *cis*ZR or *trans*ZR) or auxin (0.1 μM NAA) alone caused reduction of chlorophyll accumulation in *Arabidopsis* seedlings. Comparing *cis* and *trans* isomers effects, cultivation of seedlings on medium with *trans*ZR resulted in lower chlorophyll amounts than found with *cis*ZR (by ca. 30%). Combination of CK and NAA pronounced impairment of chlorophyll levels even more (Figure 20). Unexpectedly, excess of *cis*ZR impaired the chlorophyll contents more efficiently than *trans*ZR when applied in combination with NAA. The chlorophyll contents of *cis*ZR+NAA combination being even lower (by ca. 10%) compared to *trans*ZR+NAA variant.

Decreased accumulation of chlorophyll caused by combination of CK and NAA is obviously dependent on ethylene production. It can be deduced from the observation that the differences between effects of both zeatin isomers on NAA grown seedlings were diminished after application of an inhibitor of ethylene biosynthesis aminoethyoxyvinyl glycine (AVG, 2 μM, Figure 20).

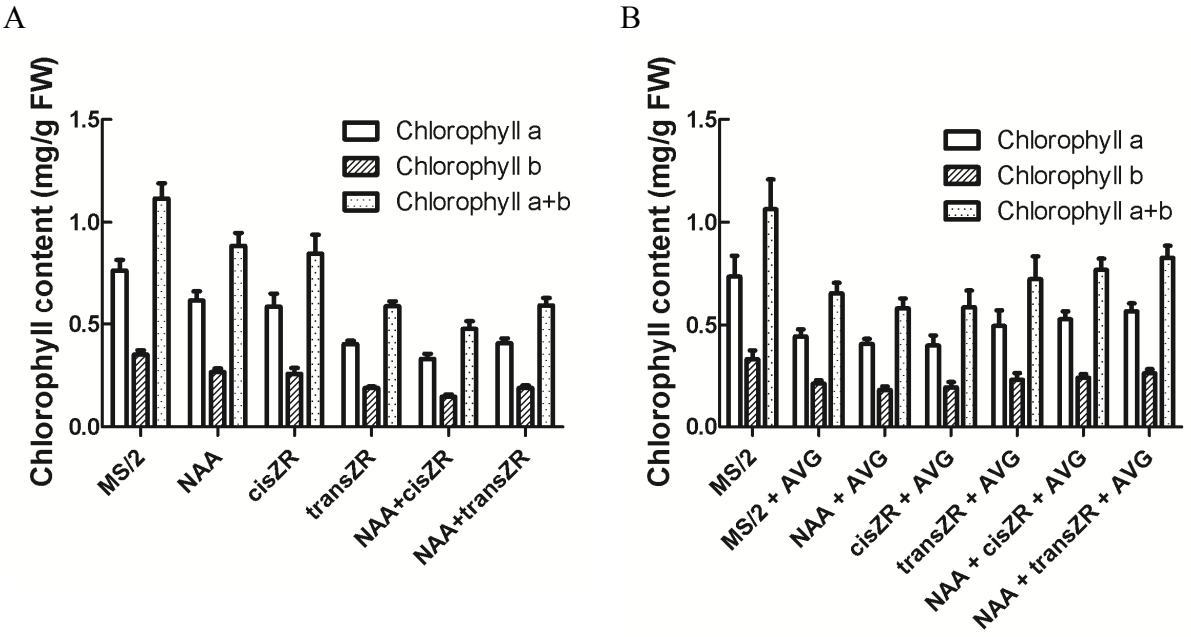


Figure 20: Chlorophyll content in shoots od 7 days old *Arabidopsis thaliana* seedlings. The seedlings were grown on media supplied with particular CK (*cis*ZR or *trans*ZR; 0,5 µM) and/or auxin (NAA; 0,1 µM). The figures depict situation without (A) and with (B) AVG (2 µM). Error bars represent standard deviation.

5.5 Effects of exogenously applied *cis*- and *trans*-zeatin isomers on root growth in *Arabidopsis thaliana*

In parallel with above experiments, seedlings of *Arabidopsis thaliana* (7 days old) were also used to determine the effects of *cis*- and *trans*ZR, applied either alone or in combination with auxin NAA, on the root growth. Addition of any of the two isomers alone resulted in an inhibition of the root growth, *trans*ZR being more effective (root length reduction by 42 %) compared to *cis*ZR (root length reduction by 16 %; Figure 22). Combination of NAA with *trans*ZR as well as with *cis*ZR caused even stronger reduction of the root growth inhibition (by 56% and 31%, respectively, compared to the control).

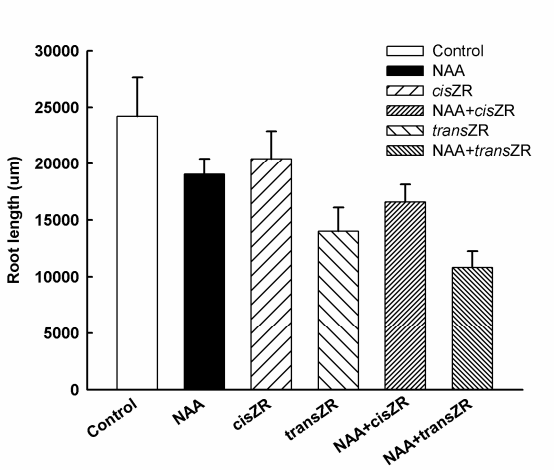


Figure 21: Effect of *cis*ZR and *trans*ZR and auxin on primary root enlargement. The seedlings were grown on media supplied with particular CK (*cis*ZR or *trans*ZR; 0,5 µM) and/or auxin (NAA; 0,1 µM). Error bars represent standard deviation.

It is known that CKs can influence auxin transport (Pernisova *et al.*, 2009). With this respect, effects of zeatin isomers upon auxin transport and subsequent auxin response were inspected and *Arabidopsis thaliana* seedlings harboring auxin responsive promoter fused to marker β-glucoronidase gene DR5:GUS were utilized.

Highest expression of DR5:GUS was detected in plants grown in the presence of NAA. Both zeatin isomers were able to reduce expression of reporter gene but *trans*ZR was more effective than *cis*ZR. The differences were even more prominent when CKs were applied simultaneously with NAA. Whereas *cis*ZR in combination with NAA only slightly reduced signal in root meristematic zone and had almost none visible effect on root tip, *trans*ZR minimized DR5:GUS expression in the meristematic zone and also weakened the signal in the root tip.

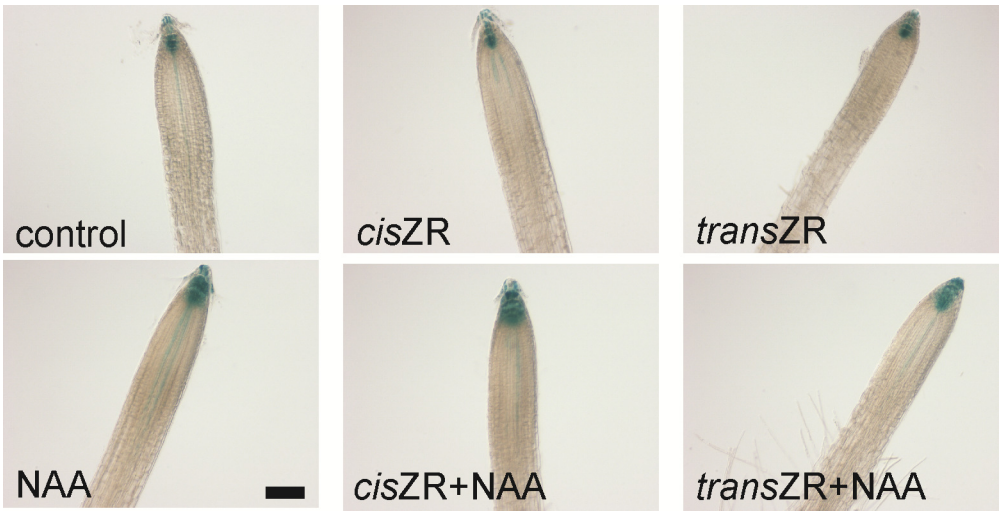


Figure 22: Details of 5 days old *Arabidopsis thaliana* roots expressing auxin response reporter gene DR5:GUS. Seedlings were grown in the presence of CK *cis*ZR or *trans*ZR (0,5 µM) and auxin NAA (0,1 µM) and their influence on auxin response in root tip is depicted. Bar represents 100 µm.

5.6 Analysis of tRNA-IPT mutants

Two *Arabidopsis thaliana* tRNA-IPTs, AtIPT2 and AtIPT9, represent biosynthetic enzymes responsible for *cis*Z formation (Miyawaki *et al.*, 2006). So far, only scarce data have been available concerning phenotypic analysis of tRNA-IPT mutants, which may provide additional information about *cis*Z function *in planta.* Thus, T-DNA insertional mutants of tRNA-IPTs, *atipt2*, *atipt9* and *atipt2 9*, were examined with emphasis on double mutant for identification of eventual phenotypic features related to *cis*Z deficiency.

5.6.1 Shoot and root growth of tRNA-IPT mutants

While the shoot and root growth of *atipt2* mutant remained almost unchanged compared to the control, significant alterations were found in *atipt9* and *atipt2 9* mutants. Both shoots and roots were considerably reduced in *atipt9* as well as in *atipt2 9.* The data of shoot and root growth analysis are shown in Figure 23.

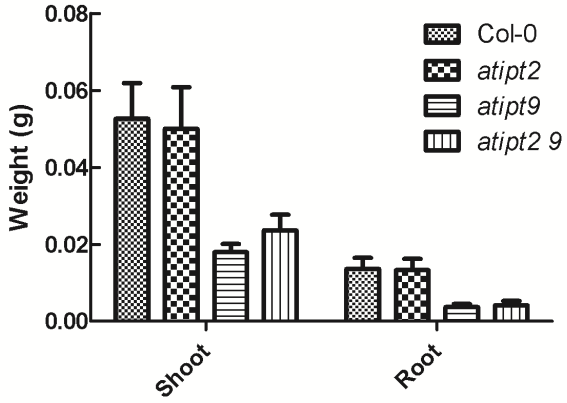


Figure 23: Shoot and root growth analysis of wild-type Col-0 and *atipt2*, *atipt9* and *atipt2 9* mutants. Shoot and root growth was analysed on the fresh weight basis of the tissues pooled from 10 seedlings. Error bars represent standard deviation.

5.6.2 Dry seeds of Arabidopsis tRNA-IPT mutants

Image analysis of tRNA-IPT mutants revealed altered seed size parameters of the *atipt2 9* mutant. In contrast to the single mutants, the dry seeds of the double mutant were slightly smaller, with reduced length and width (by ca. 12% and 25%, respectively, compared to the wild-type Col-0 (Figure 24) and sometimes shriveled (Figure 25).

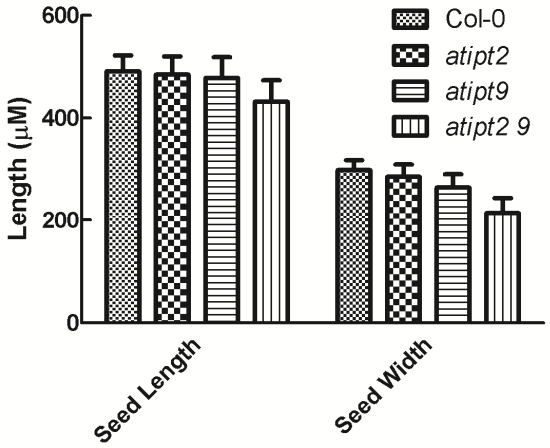


Figure 24: The dry seed size parameters of tRNA-IPT *atipt2*, *atipt9* and *atipt2 9* mutants. Error bars represent standard deviation.

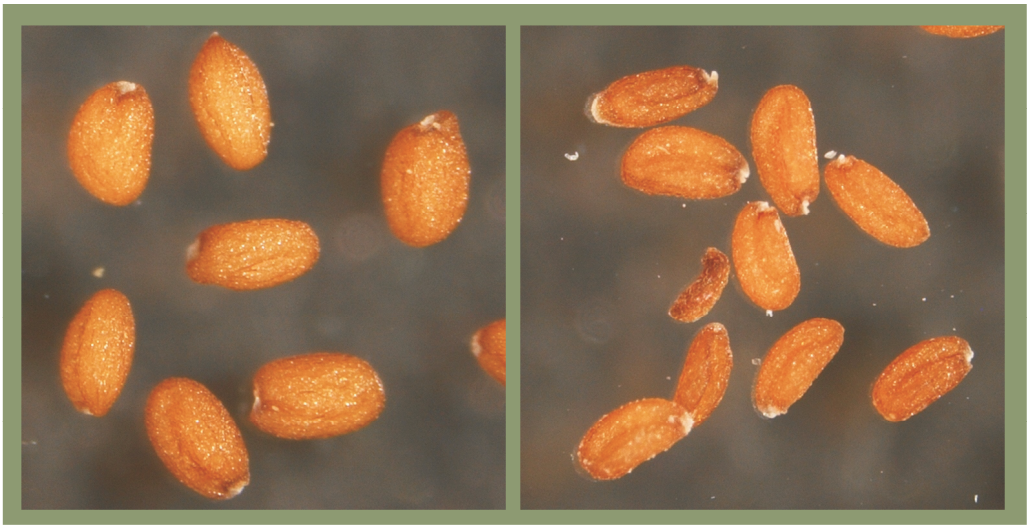


Figure 25: Images of *Arabidopsis thaliana* dry seeds. Wild-type Col-0 (left panel), *atipt2 9* double mutant (right panel). Both images are taken under the same magnification.

5.6.3 Phenotypic analysis of atipt2 9 double mutant

5.6.3.1 Effect of sucrose deficiency in cultivation medium on the root growth of atipt2 9 double mutant

Various mutant phenotypes can be rescued by the sucrose to a certain extent, which is utilized as a source of energy and metabolites (Kim *et al.*, 2004). Phenotype of the *atipt 2 9* double mutant was emphasized on the growth medium lacking sucrose. In order to detect additinal growth defetcs *atipt2 9* mutant seedlings were cultivated on sucrose-free medium. While the lack of sucrose did not cause any changes in the root length of the wild-type seedlings, it resulted in strong reduction of the root growth of *atipt 2 9* mutant (Figure 26).

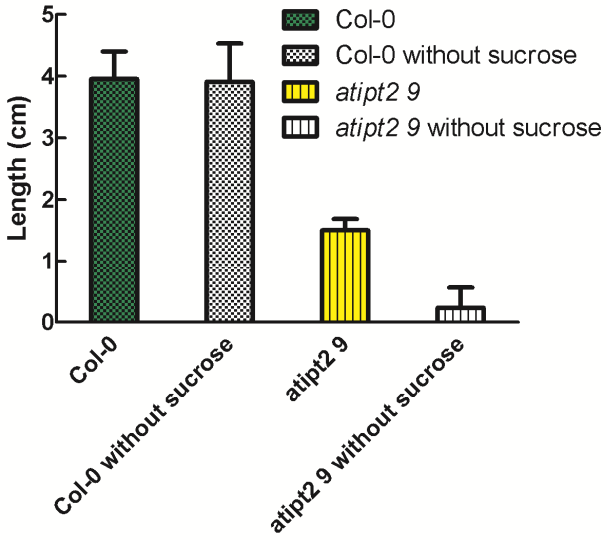


Figure 26: Comparison of the root growth response of wild-type and *atipt2 9* mutant in the presence and absence of sucrose in medium. Error bars represent standard deviation.

5.6.3.2 Chlorophyll content in shoots and leaves of atipt2 9 mutant

Shoots and leaves of *atipt2 9* mutant were chlorotic and the chlorophyll levels were reduced almost to 50% of those found in wild-type plants (Figure 27). Chlorosis of the youngest *atipt2 9* leaves partially diminished during their development. Greening of the tissues proceeded gradually from the tips to the leaf bases (Figure 28).

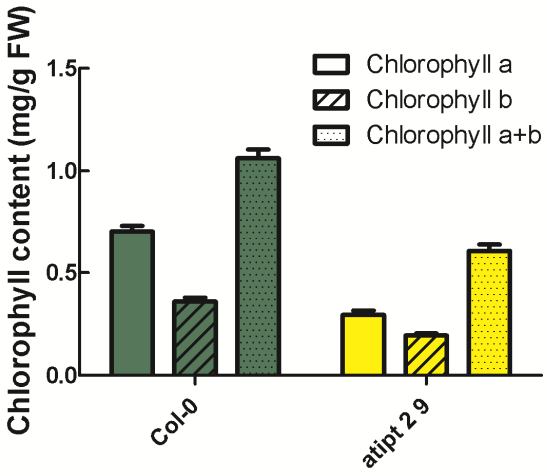


Figure 27: Chlorophyll content in shoots and leaves of wild-type Col-0 and *atipt2 9* double mutant. Error bars represent standard deviation.

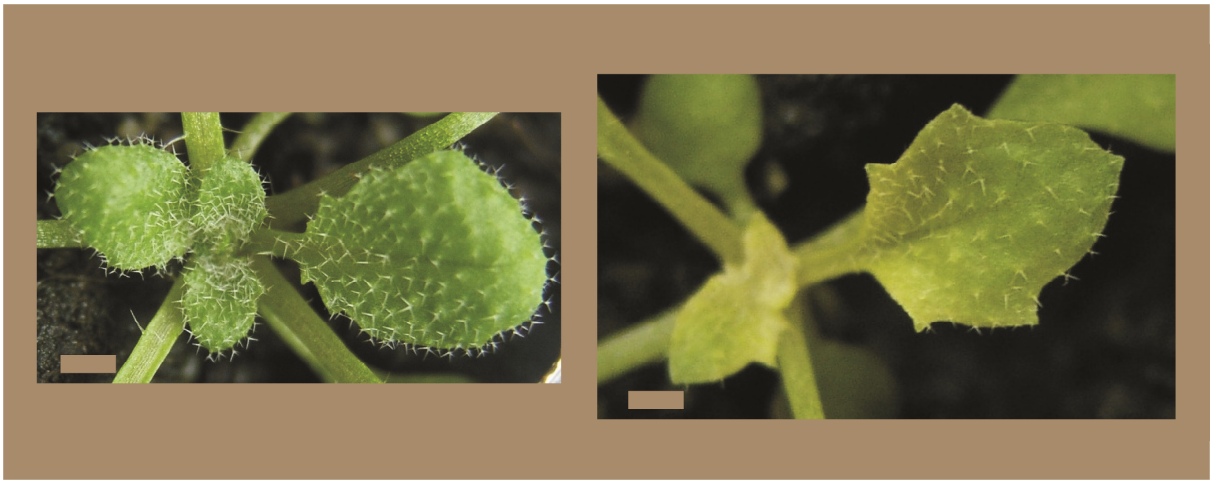


Figure 28: Details of *Arabidopsis thaliana* true leaf shapes at the beginning of their formation. 21-days old seedlings of wild-type Col-0 (left panel) and *atipt 2 9* double mutant (right panel) are shown. Plants were grown in soil. Bar represents 1 mm.

6 Discussion

*cis*Z-type CKs have been considered insignificant CK since their discovery. In spite of their low biological activity number of reports previously demonstrated a high abundance of free *cis*Z and/or its derivatives in algae (Stirk *et al.*, 2003; Ördög *et al.*, 2004), mosses (Von Schwartzenberg *et al.*, 2007) and various seed plants including monocots (Parker *et al.*, 1989; Veach *et al.*, 2003) and dicots (Stirk *et al.*, 2008; Malkawi *et al.*, 2007; Ananieva *et al.*, 2004; Dobrev *et al.*, 2002; Emery *et al.*, 1998; Emery *et al.*, 2000). The herein described data and our recently published screen through the evolution of land plants (Gajdošová *et al.*, 2011) together with the identification of genes and enzymes specific for *cis*Z metabolic reactions (Martin et al., 2001; Veach et al., 2003) as well as recognition of *cis*Z-type CKs by CK receptors (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004), endorse the hypothesis that *cis*Z-type CKs occur in the plant kingdom ubiquitously being more prevalent and more relevant to CK biology than previously thought.

Although *cis*Z is probably evolutionary older than its *trans* counterpart, inferring from *cis*ZR presence in tRNA (Hall *et al.*, 1967), prevalence of *cis*Z derivatives does not seem to have a direct connection to evolutionary complexity. Only representatives of *Poaceae* family have been found in our study to show relatively consistent majority of *cis*Z-type CKs. These findings are in agreement with previous reports demonstrating abundance of *cis*Z and/or its derivatives in most members of this family (Parker *et al.*, 1989; Takagi *et al*., 1989 and Veach *et al.*, 2003 for wheat, rice and maize). It was also published 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). On the basis of these data it was proposed that *cis*Zs occurence in *Poaceae* might emanate from specific ontogeny (Gajdosova *et al.*, 2011).

Lately, rapid accumulation of *cis*ZRMP has been detected 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 also observed in plants exposed to heat (Dobra *et al.*, 2010) or biotic stress (Pertry *et al.*, 2009) and after administration of inhibitors limiting growth (Blagoeva *et al.*, 2003; Blagoeva *et al.*, 2004). Lower seed dormancy of annual rye grass was accompanied with higher levels of *cis*Z (Goggin *et al.*, 2010). Likewise, reduction of *cis*ZR levels in buds of chickpea was recorded after decapitation (Mader *et al.*, 2003) and probably facilitates release of buds from dormancy. High levels of *cis*Z-type CKs were also found in *Mercurialis* associated with the induction of male sterility (Louis *et al.*, 1990).

Recent data indicated that potato hinders vegetative growth of aerial part during tuberization (Fischer et al., 2008). Therefore, *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 tissues to ensure allocation of more nutrients to stolons for tuber formation. Also *cis*ZR accumulation at the end of embryogenesis in developing pea embryos (Quesnelle and Emery, 2007) might be associated with ceased growth of embryo. Additionally, *cis*ZR has a high resistance against degradation by AtCKX2, the *Arabidopsis* CKX isoform that is expressed during senescence (Gajdosova *et al.*, 2011). Taken together, all these states are obviously associated with growth-limiting conditions resulting from internal or external cues and *cis*Z-type CKs may have a crucial role on these occasions.

Based on the above mentioned data it is suggested that the *cis*Z function might reside in maintenance of 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 finished. In this regard, *cis*Z isomer and/or its derivatives may be relevant under growth-limiting conditions associated with a developmental program or external signals leading to plant optimal survival via reduction but not complete cessation of CK signalling (Gajdosova *et al.*, 2011). This hypothesis does not, however, exclude (a) potential role(s) of cis-zeatins in regulation of other physiological processes in plants.

Wide-spread occurrence of free *cis*Z-type CKs implies their physiological function. Here, biological activity of *cis*Z and its derivatives was demonstrated in tobacco callus and chlorophyll retention bioassays. Our results prove that *cis*Z-type CKs are able to trigger cell division and growth of CK-dependent tobacco callus. The optimal cell-division stimulatory concentrations of both *cis*- and *trans*Z free bases found in our experiments are comparable with those reported by Schmitz *et al.* (1972). Interestingly, *cis*ZR showed maximal effect among all tested *cis*Z derivatives being active in the same concentration as its *trans* counterpart. Another set of data reveals that *cis*Z-type CKs are able to delay the dark-induced chlorophyll degradation in oat and wheat leaves. Their activity of *cis*-zeatins was generally lower than that of corresponding *trans*-isomers. The exception to this statement was again *cis*ZR as the most effective CK among all tested *cis*Z derivatives and only slightly less active than its *trans* counterpart. Data demonstrating high bioactivities of CK ribosides confronted with other CK forms were reported also by other studies (Doležal *et al.*, 2007; Kamínek *et al.*, 1979; Kaminek *et al.*, 1987; Tarkowská *et al.*, 2003). High *cis*ZR activity in bioassays might reside in the more efficient transport of CK ribosides and/or in lower degradation by CKX

(Sakakibara, 2006). It also hints a preference for *cis*ZR rather than *cis*Z in the mediation of the extracellular signal transduction to influence physiological effect(s).

Glycosylation of CKs may occur either on the purine ring or on the side chain of the CK molecule (Vaňková 1999, Sakakibara 2006). From the physiological point of view the crucial aspect is if this process is irreversible yielding stable detoxification or inactivation products (putatively N7- and N9-glucosides) or reversible allowing release of free CK bases or ribosides from the storage forms (O-glucosides, N3-glucosides). Our data from oat chlorophyll retention and tobacco callus bioassays confirmed previously reported biological activity of CK-O-glucosides. However, rather unexpectedly N9-glucosides of *trans*Z (and in much lesser extent of *cis*Z as well) have been also observed to effectively counteract chlorophyll degradation in oat leaf segments. The efficiency of CK-N-glucosides in CK bioassays was reported also by some other authors (Mik *et al.*, 2011; Upfold and Vanstaden, 1990). The experiment testing *cis*Z9G and *trans*Z9G stability in solution excluded possibility of spontaneous decay of N9-glucosides to free bases. Considering the additional fact, that cleavage of O- and N3-glucosides by β-glucosidases was proven (Brzobohaty *et al.*, 1993) there might also exist a unique metabolic pathway responsible for cleaving glucose at N9 position and releasing biologically active free base. Though spontaneous release of free CK bases or ribosides from their N-glycoconjugates proceeding in plant tissues under developmental requirements cannot be completely excluded. CK-N-glucosides may be exposed to a number of various physicochemical conditions such as e.g. strong aqueous acids facilitating hydrolysis of glycosidic bond, but this type of hydrolysis seems to occur only in very limited range thus being physiologically insignificant.

It is known that *cis*Z is able to interact with an AHK3 receptor (Romanov *et al.*, 2006; Spíchal *et al.*, 2004). The AHK3 signal perception responsive to *cis*Z and induction of ARR5 expression in *Arabidopsis* (Gajdosova *et al.*, 2011) confirmed a relevant CK signalling cascade evoked by *cis*Z. Recently published data indicate that *cis*Z might influence – probably by means of competition with other more active CKs on AtAHK3 receptor – the transport of auxin (Pernisova *et al.*, 2009) and thus could elicit responses resulting in the preservation of only essential physiological processes. Nevertheless, possible crosstalk of *cis*Z-type CKs with other phytohormones via CK signalling components cannot be dismissed.

The most important information gained from metabolic studies resides in unobserved conversion of labeled *cis* into *trans* isomer and vice versa regardless the short- or long-term feeding experiment. It might question 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 the conditions or type of material used here might not be sufficient to provoke its activity. In general, the metabolic fate of both isomers differs. Our study revealed that various species exploit different ways to deactivate CKs via glucosylation. In *Arabidopsis* genome, 5 out of 102 putative glucosyltransferases were found to be able to add sugar residues to hydroxyl group of both *trans*- and *cis*Z isomers as well as to N7- and N9-nitrogens on the purine ring (Hou et al., 2004). On the other hand, two characterized maize *O*-glucosyltransferases are closely specific only to *cis*Z isomer (Martin et al. 2001, Veach et al. 2003), which accords with high abundance of *cis*Z-type CKs in monocotyledonous plants. Supporting these data, *O*-glucosylation was identified as a preferential metabolic pathway of *cis*Z in oat leaves in our experiments. However, endogenous N7- and N9-glucosides have never been detected in oat tissues or have been found to comprise only a very negligible portion of total CK pool. It is probable that the zeatin glucosylation has been acquired independently in different plant families during evolution and some pathways in some species could be missing. Tobacco, the representative of dicotyledonous plants from Solanaceae family used in our study, exhibits different metabolic pattern than oat as a representative monocotyledonous species. While *trans*Z was glucosylated in tobacco cells both at the side chain as well as at the purine ring, *cis*Z was metabolized relatively simply being not glucosylated at all or only very irrelevantly on N7-position within short-term incubation. Thus, the short-term metabolism didn't display any glucosylation of *cis*Z but presence (and even prevalence) of *cis*Z7G in tobacco leaf samples indicates possible time lag for *O*- and *N*-glucosylation of *cis*Z, which might have been also a result of non-specific glucosylation reaction.

Degradation of CKs in the CKX assay revealed a similar affinity of the oat leaf enzymes to both zeatin isomers. This outcome is consonant with results of metabolic analysis in which similar amounts of degradation products occurred. The fact that tobacco CKX has higher affinity to *cis*Z than to *trans*Z in the *in vitro* assays explains substantially higher amount of degradation products, adenine and adenosine, found as metabolic products of exogenous *cis*Z *in vivo*. This indicates more important role for CKX in degradation of *cis*Z than *trans*Z isomer in tobacco. It is possible that special structural features of the enzyme active center determine the better access of *cis*Z than *trans*Z to the catalytic site of, at least some, tobacco CKXs (von Schwartzenberg *et al.*, 2007). Low affinity of CKX to *cis*Z in dry seeds of oat, maize and lucerne might be related to preservation of weakly active CK forms in tissues with limited cell division and growth.

To summarize the data from metabolic experiments, it can be concluded that both isomers of zeatin can be *N*- or *O*-glucosylated and degraded by CKX activity in a species-specific manner in oat and wheat. It is obvious that *cis*Z is metabolised separately from *trans*Z. Recent evidence for importance of tRNA-dependent CK biosynthesis in moss *Physcomitrella* could provide feasible interpretation (Yevdakova *et al.*, 2008). Evolutionary older species such as *Physcomitrella* and family *Poaceae* might have preserved pristine modifications of *cis*Z-type CKs while evolutionary younger species have developed a new strategy. As *cis*Z ↔ *trans*Z isomerization does not appear to play a major role in CK interconversions, this highlights the need to study the two CK isomer systems in plants independently, especially with a view to the recent demonstration of distinct *cis*- and *trans*Z biosynthetic origins (Kasahara et al. 2004).

In search for *cis*Z-specific function effects of *cis*ZR on primary root growth in the presence of auxin were examined. It is known that CKs and auxin are able to retard root enlargement (Beemster and Baskin, 2000; Ruzicka *et al.*, 2009). Results showed that *cis*ZR is less effective in inhibition of primary root elongation compared to *trans*Z. The supplementation of auxin (NAA) to the media together with CKs caused more pronounced growth retardation in similar trend as CKs alone. It might mean that both isomers are capable to inhibit root growth by presumably same pathway, plausibly via modification of auxin transport, but with diverse efficiency. This presumption is supported by the corresponding data depicting correspoding changes in reduction of auxin response in primary root tips after cultivation with all combinations of tested hormones.

CKs are able to influence chlorophyll degradation but also its biosynthesis in plants (Jelic and Bogdanovic 1989). Exogenously applied auxins were found to reduce chlorophyll formation by suppressing the development of thylakoid membranes (Sunderland and Wells 1968). High levels of auxin are known to enhance ethylene biosynthesis (Yu and Yang, 1979). Similarly, CKs are able to induce ethylene biosynthesis (Chae *et al.*, 2003) and ethylene is necessary for induction of chlorophyll degradation by chlorophyllase (Purvis and Barmore, 1981). Therefore, impact of both zeatin isomers on crosstalk between auxin and CK actions on chlorophyll levels via ethylene biosynthesis were examined. Experiments revealed unexpectedly more pronounced decrease of chlorophyll accumulation in *Arabidopsis* seedlings after cultivation on media supplied with *cis*ZR compared to *trans*ZR. As AVG, the inhibitor of ethylene biosynthesis, counteracted differences in the effects after administration of both Z isomers to NAA, it seems probable that *cis*ZR and *trans*ZR might affect chlorophyll accumulation and/or degradation differentially by means of ethylene biosynthesis. However,

the actions of CKs and NAA together did not show normal additive effects, as it was preliminarily inferred after the use of single compounds. It might be possible that each Z isomer could affect a slightly different route to influence ethylene biosynthesis and subsequently reduction of chlorophyll content.

The tRNA-IPT mutants represent a suitable tool for studying potential *cis*Z functions *in planta*, however, the existing knowledge concerning their phenotypic traits is still rather limited. Our analysis of *Arabidopsis* tRNA-IPT mutants *atipt2*, *atipt9* and *atipt2 9* affirmed previously reported chlorosis (Miyawaki *et al.*, 2006) and retarded growth connected with nonfunctional AtIPT9 (Vadassery *et al.*, 2008). The chlorosis of young leaves of *atipt2 9* mutant was partially rescued from the leaf tip to the base during their development. CKs were found to stimulate chloroplast differentiation and zeatin treatment induced formation of a greater number of chloroplasts (de Oliveira *et al.*, 2008). The double mutant apparently lacks CKs for optimal leaf photomorphogenesis, which is indicated by indispensable involvement of light in its greening and suggests requirements of tRNA-derived CKs for a proper plastid development. Aggravated growth of the *atipt2 9* double mutant was observed in the absence of sucrose in growth medium. Considering impaired tRNA prenylation in the double mutant, translational defects may result in higher demand for energy to sustain necessary protein translation and finally cause the growth retardation. According to Miyawaki *et al.* (2006), mutation in AtIPT9 leads to lowering of iP, *cis*Z and *trans*Z derivatives abundance. As the biological activity of iP and *trans*Z derivatives is high, they are likely to be responsible for overall CK defects. Although the double mutant was *cis*Z deficient, it apparently suffered more from defects in proteosynthesis and reduction of iP- and *trans*Z-type CKs levels than from those of *cis*Z.

CKs are known to influence leaf shape via interaction with auxin signalling (Shani *et al.*, 2010), which is a crucial phenomenon in determination of leaf margin shape (Bilsborough *et al.*, 2011). Therefore, slightly altered shape of true leaves in *atipt2 9* double mutant might be a consequence of changed balance between CKs and auxin in leaf marginal blastozone. CKs can positively influence sink strength (Mok, 1994). Therefore the modified shape of *atipt2 9* seeds might be attributed to reduced seed filling as assumed from their shriveled shape and reduced CK levels (Miyawaki *et al.*, 2006). Other explanations are also possible. For example, the low seed filling of the mutant might result from its overall proteosynthetic defects. Such a seriously increased demand on energy might be followed by decreased ability to form normal storage structures including seeds. These results imply that *atipt2 9* double mutant might not be the most suitable material for studying of *cis*Z deficiency. Probably

atipt2 mutant might represent a more appropriate material as none prominent phenotype was reported but it has significantly reduced levels of *cis*Z-type CKs (Miyawaki *et al.*, 2006).

To summarize, *cis*Z-type CKs in plants occur ubiquitously in the plant kingdom, posses biological activity. Both zeatin isomers differ in their metabolism. In addition, *cis*Z might act via ethylene by different pathway in contrast to *trans*Z in some occasions. All these results together with *cis*Z affinity to some CK receptors clearly indicate unique physiological function of *cis*Z derivatives, which may probably consist in a delicate regulation of CK response(s) and maintenance of minimal CK activity under growth-limiting conditions plausibly related to certain tissues, developmental stages or environmental conditions. However, possible role(s) of *cis*Z-type CKs in control of other physiological processes in plants cannot be exluded.

7 References

Akiyoshi DE, Klee H, Amasino RM, Nester EW, Gordon MP. 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-Biological Sciences* **81**, 5994-5998.

Akiyoshi DE, Morris RO, Hinz R, Mischke BS, Kosuge T, Garfinkel DJ, Gordon MP, Nester EW. 1983. Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **80**, 407-411.

Allen M, Qin WS, Moreau F, Moffatt B. 2002. Adenine phosphoribosyltransferase isoforms of Arabidopsis and their potential contributions to adenine and cytokinin metabolism. *Physiologia Plantarum* **115**, 56-68.

Ananieva K, Ananiev ED, Doncheva S, Georgieva K, Tzvetkova N, Kaminek M, Motyka V, Dobrev P, Gajdosova S, Malbeck J. 2008. Senescence progression in a single darkened cotyledon depends on the light status of the other cotyledon in Cucurbita pepo (zucchini) seedlings: potential involvement of cytokinins and cytokinin oxidase/dehydrogenase activity. *Physiologia Plantarum* **134**, 609-623.

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

Anantharaman V, Aravind L. 2001. The CHASE domain: a predicted ligand-binding module in plant cytokinin receptors and other eukaryotic and bacterial receptors. *Trends in Biochemical Sciences* **26**, 579-582.

Argyros RD, Mathews DE, Chiang YH, Palmer CM, Thibault DM, Etheridge N, Argyros DA, Mason MG, Kieber JJ, Schaller GE. 2008. Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. *Plant Cell* **20**, 2102-2116.

Armstrong D. 1994. Cytokinin oxidase and the regulation of cytokinin degradation. In: Mok D, Mok M, eds. *Cytokinins. Chemistry, Activity, and Function.*: CRC Press, 139-154.

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.

Beemster GTS, Baskin TI. 2000. STUNTED PLANT 1 mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of arabidopsis. *Plant Physiology* **124**, 1718-1727.

Bilsborough GD, Runions A, Barkoulas M, Jenkins HW, Hasson A, Galinha C, Laufs P, Hay A, Prusinkiewicz P, Tsiantis M. 2011. Model for the regulation of Arabidopsis thaliana leaf margin development. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 3424-3429.

Bilyeu KD, Cole JL, Laskey JG, Riekhof WR, Esparza TJ, Kramer MD, Morris RO. 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. *Plant Physiology* **125**, 378-386.

Bjork GR, Durand JMB, Hagervall TG, Leipuviene R, Lundgren HK, Nilsson K, Chen P, Qian Q, Urbonavicius J. 1999. Transfer RNA modification: influence on translational frameshifting and metabolism. *Febs Letters* **452**, 47-51.

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.

Broo A, Holmen A. 1996. Ab initio MP2 and DFT calculations of geometry and solution tautomerism of purine and some purine derivatives. *Chemical Physics* **211**, 147-161.

Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K. 1993. Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. *Science* **262**, 1051-1054.

Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K. 1993. Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. *Science* **262**, 1051-1054.

Connolly DM, Winkler ME. 1989. Genetic and physiological relationships among the miaA gene, 2-methylthio-N6-(delta 2-isopentenyl)-adenosine tRNA modification, and spontaneous mutagenesis in Escherichia coli K-12. *Journal of Bacteriology* **171**, 3233-3246.

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

D'Agostino IB, Kieber JJ. 1999. Phosphorelay signal transduction: the emerging family of plant response regulators. *Trends in Biochemical Sciences* **24**, 452-456.

de Oliveira LM, Paiva R, Ferreira de Santana JR, Alves E, Nogueira RC, Pereira FD. 2008. Effect of cytokinins on in vitro development of autotrophism and acclimatization of Annona glabra L. *In Vitro Cellular & Developmental Biology-Plant* **44**, 128-135.

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.

Dixon SC, Martin RC, Mok MC, Shaw G, Mok DWS. 1989. Zeatin Glycosylation Enzymes in Phaseolus. Isolation of O-Glucosyltransferase from *P. lunatus* and Comparison to O-Xylosyltransferase from *P. vulgaris*. *Plant Physiology* **90**, 1316-1321.

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. *J Chromatogr 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.

Einset JW, Swaminathan S, Skoog F. 1976. Ribosyl-*cis*-zeatin in a leucyl transfer RNA species from peas. *Plant Physiology* **58**, 140-142.

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.

Entsch B, Parker CW, Letham DS, Summons RE. 1979. Preparation and characterization, using high-performance liquid chromatography, of an enzyme forming glucosides of cytokinins. *Biochimica Et Biophysica Acta* **570**, 124-139.

Entsch B, Parker CW, Letham DS. 1983. An enzyme from lupin seeds forming alanine derivatives of cytokinins. *Phytochemistry* **22**, 375-381.

Esberg B, Leung HCE, Tsui HCT, Bjork GR, Winkler ME. 1999. Identification of the miaB gene, involved in methylthiolation of isopentenylated A37 derivatives in the tRNA of Salmonella typhimurium and Escherichia coli. *Journal of Bacteriology* **181**, 7256-7265.

Ferreira FJ, Kieber JJ. 2005. Cytokinin signaling. *Current Opinion in Plant Biology* **8**, 518-525.

Frebort I, Kowalska M, Hluska T, Frebortova J, Galuszka P. 2011. Evolution of cytokinin biosynthesis and degradation. *Journal of Experimental Botany* **62**, 2431-2452.

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

Galuszka P, Popelkova H, Werner T, Frebortova J, Pospisilova H, Mik V, Kollmer I, Schmulling T, Frebort I. 2007. Biochemical characterization of cytokinin Oxidases/Dehydrogenases from Arabidopsis thaliana expressed in Nicotiana tabacum L. *Journal of Plant Growth Regulation* **26**, 255-267.

Garfinkel DJ, Simpson RB, Ream LW, White FF, Gordon MP, Nester EW. 1981. Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. *Cell* **27**, 143-153.

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.

Gelvin SB. 1990. Crown gall disease and hairy root disease : a sledgehammer and a tackhammer. *Plant Physiology* **92**, 281-285.

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.

Golovko A, Hjalm G, Sitbon F, Nicander B. 2000. Cloning of a human tRNA isopentenyl transferase. *Gene* **258**, 85-93.

Golovko A, Sitbon F, Tillberg E, Nicander B. 2007. Expression of a human tRNA isopentenyltransferase in tobacco reveals a developmental role for tRNA isopentenyladenosine. *Functional Plant Biology* **34**, 654-661.

Hall RH, Csonka L, David H, McLennan B. 1967. Cytokinins in the soluble RNA of plant tissues. *Science* **156**, 69-71.

Halmann M. 1990. Synthetic plant growth regulators. *Advances in Agronomy* **43**, 47-105.

Hanus J, Siglerova V, Matucha M. 2000. N-6-alkyladenosines and adenines labelled with tritium. *Journal of Labelled Compounds & Radiopharmaceuticals* **43**, 523-531.

Holtz J, Klambt D. 1975. tRNA isopentenyltransferase from *Lactobacillus acidophilus* ATCC 4963. *Hoppe-Seylers Zeitschrift Fur Physiologische Chemie* **356**, 1459-1464.

Holtz J, Klambt D. 1978. tRNA isopentenyltransferase from *Zea mays* L. Characterization of the isopentenylation reaction of tRNA, oligo (A) and other nucleic acids. *Hoppe-Seylers Zeitschrift Fur Physiologische Chemie* **359**, 89-101.

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.

Horgan R, Hewett EW, Purse JG, Wareing PF. 1973. A new cytokinin from *Populus x robusta*. *Tetrahedron Letters*, 2827-2828.

Hou B, Lim EK, Higgins GS, Bowles DJ. 2004. N-glucosylation of cytokinins by glycosyltransferases of Arabidopsis thaliana. *Journal of Biological Chemistry* **279**, 47822-47832.

Hutchison CE, Li J, Argueso C, Gonzalez M, Lee E, Lewis MW, Maxwell BB, Perdue TD, Schaller GE, Alonso JM, Ecker JR, Kieber JJ. 2006. The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *Plant Cell* **18**, 3073-3087.

Hwang D, Chen HC, Sheen J. 2002. Two-component signal transduction pathways in Arabidopsis. *Plant Physiology* **129**, 500-515.

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.

Chen CM, Hall RH. 1969. Biosynthesis of N6-([delta] 2-isopentenyl) adenosine in the transfer ribonucleic acid of cultured tobacco pith tissue. *Phytochemistry* **8**, 1687-&.

Chen CM. 1997. Cytokinin biosynthesis and interconversion. *Physiologia Plantarum* **101**, 665-673.

Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Kiba T, Ueguchi C, Sugiyama T, Mizuno T. 1999. Compilation and characterization of Arabidopsis thaliana response regulators implicated in His-Asp phosphorelay signal transduction. *Plant and Cell Physiology* **40**, 733-742.

Imamura A, Hanaki N, Umeda H, Nakamura A, Suzuki T, Ueguchi C, Mizuno T. 1998. Response regulators implicated in His-to-Asp phosphotransfer signaling in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2691-2696.

Imamura A, Yoshino Y, Mizuno T. 2001. Cellular localization of the signaling components of Arabidopsis His-to-Asp phosphorelay. *Bioscience Biotechnology and Biochemistry* **65**, 2113-2117.

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T. 2001. Identification of CRE1 as a cytokinin receptor from Arabidopsis. *Nature* **409**, 1060-1063.

Ishida K, Yamashino T, Mizuno T. 2008. Expression of the Cytokinin-Induced Type-A Response Regulator Gene ARR9 Is Regulated by the Circadian Clock in Arabidopsis thaliana. *Bioscience Biotechnology and Biochemistry* **72**, 3025-3029.

Jelic G, Bogdanovic M. 1989. Antagonism between abscisic acid and cytokinin in chlorophyll synthesis in pine seedlings. *Plant Science* **61**, 197-202.

Kakimoto T. 1996. CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* **274**, 982-985.

Kakimoto T. 2001. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate : ATP/ADP isopentenyltransferases. *Plant and Cell Physiology* **42**, 677-685.

Kakimoto T. 2003. Biosynthesis of cytokinins. *Journal of Plant Research* **116**, 233-239.

Kamada-Nobusada T, Sakakibara H. 2009. Molecular basis for cytokinin biosynthesis. *Phytochemistry* **70**, 444-449.

Kaminek M, Armstrong DJ. 1990. Genotypic variation in cytokinin oxidase from Phaseolus callus cultures. *Plant Physiology* **93**, 1530-1538.

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.

Kaminek M, Vanek T, Motyka V. 1987. Cytokinin activities of N-6-bensyladenosine derivatives hydroxylated on the side-chain phenyl ring. *Journal of Plant Growth Regulation* **6**, 113-120.

Kaminska KH, Baraniak U, Boniecki M, Nowaczyk K, Czerwoniec A, Bujnicki JM. 2008. Structural bioinformatics analysis of enzymes involved in the biosynthesis pathway of the hypermodified nucleoside ms(2)io(6)A37 in tRNA. *Proteins-Structure Function and Bioinformatics* **70**, 1-18.

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.

Kaul S, Koo HL, Jenkins J, Rizzo M, Rooney T, Tallon LJ, Feldblyum T, Nierman W, Benito MI, Lin XY, Town CD, Venter JC, Fraser CM, Tabata S, Nakamura Y, Kaneko T, Sato S, Asamizu E, Kato T, Kotani H, Sasamoto S, Ecker JR, Theologis A, Federspiel NA, Palm CJ, Osborne BI, Shinn P, Conway AB, Vysotskaia VS, Dewar K, Conn L, Lenz CA, Kim CJ, Hansen NF, Liu SX, Buehler E, Altafi H, Sakano H, Dunn P, Lam B, Pham PK, Chao Q, Nguyen M, Yu GX, Chen HM, Southwick A, Lee JM, Miranda M, Toriumi MJ, Davis RW, Wambutt R, Murphy G, Dusterhoft A, Stiekema W, Pohl T, Entian KD, Terryn N, Volckaert G, Salanoubat M, Choisne N, Rieger M, Ansorge W, Unseld M, Fartmann B, Valle G, Artiguenave F, Weissenbach J, Quetier F, Wilson RK, de la Bastide M, Sekhon M, Huang E, Spiegel L, Gnoj L, Pepin K, Murray J, Johnson D, Habermann K, Dedhia N, Parnell L, Preston R, Hillier L, Chen E, Marra M, Martienssen R, McCombie WR, Mayer K, White O, Bevan M, Lemcke K, Creasy TH, Bielke C, Haas B, Haase D, Maiti R, Rudd S, Peterson J, Schoof H, Frishman D, Morgenstern B, Zaccaria P, Ermolaeva M, Pertea M, Quackenbush J, Volfovsky N, Wu DY, Lowe TM, Salzberg SL, Mewes HW, Rounsley S, Bush D, Subramaniam S, Levin I, Norris S, Schmidt R, Acarkan A, Bancroft I, Brennicke A, Eisen JA, Bureau T, Legault BA, Le QH, Agrawal N, Yu Z, Copenhaver GP, Luo S, Pikaard CS, Preuss D, Paulsen IT, Sussman M, Britt AB, Selinger DA, Pandey R, Mount DW, Chandler VL, Jorgensen RA, Pikaard C, Juergens G, Meyerowitz EM, Dangl J, Jones JDG, Chen M, Chory J, Somerville MC, Ar Gen I. 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature* **408**, 796-815.

Kiba T, Yamada H, Mizuno T. 2002. Characterization of the ARR15 and ARR16 response regulators with special reference to the cytokinin signaling pathway mediated by the AHK4 histidine kinase in roots of Arabidopsis thaliana. *Plant and Cell Physiology* **43**, 1059-1066.

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 of the United States of America* **103**, 814-819.

Kim TH, Kim BH, Yahalom A, Chamovitz DA, von Arnim AG. 2004. Translational regulation via 5 ' mRNA leader sequences revealed by mutational analysis of the Arabidopsis, translation initiation factor subunit elF3h. *Plant Cell* **16**, 3341-3356.

Krall L, Raschke M, Zenk MH, Baron C. 2002. The Tzs protein from Agrobacterium tumefaciens C58 produces zeatin riboside 5 '-phosphate from 4-hydroxy-3-methyl-2-(E)-butenyl diphosphate and AMP. *Febs Letters* **527**, 315-318.

Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, Kyozuka J. 2007. Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* **445**, 652-655.

Kuzuyama T. 2002. Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. *Bioscience Biotechnology and Biochemistry* **66**, 1619-1627.

Lee DJ, Park JY, Ku SJ, Ha YM, Kim S, Kim MD, Oh MH, Kim J. 2007. Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7(ARR7) overexpression in cytokinin response. *Molecular Genetics and Genomics* **277**, 115-137.

Letham DS, Palni LMS. 1983. Cytokinin biosynthesis and metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **34**, 163-197.

Letham DS, Shannon JS, McDonald IR. 1964. The structure of Zeatin, a factor inducing cell division. *Proceedings of the Chemical Society of London*, 230-231.

Letham DS, Summons RE, Entsch B, Gollnow BI, Parker CW, Macleod JK. 1978. Regulators of cell-division in plant-tissues .26. Glucosylation of cytokinin analogs. *Phytochemistry* **17**, 2053-2057.

Letham DS. 1963. Zeatin, a factor inducing cell division isolated from *Zea mays*. *Life Sciences*, 569-573.

Lohrmann J, Harter K. 2002. Plant two-component signaling systems and the role of response regulators. *Plant Physiology* **128**, 363-369.

Louis JP, Augur C, Teller G. 1990. CYTOKININS AND DIFFERENTIATION PROCESSES 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.

Mahonen AP, Bonke M, Kauppinen L, Riikonen M, Benfey PN, Helariutta Y. 2000. A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. *Genes & Development* **14**, 2938-2943.

Mahonen AP, Higuchi M, Tormakangas K, Miyawaki K, Pischke MS, Sussman MR, Helariutta Y, Kakimoto T. 2006. Cytokinins regulate a bidirectional phosphorelay network in Arabidopsis. *Current Biology* **16**, 1116-1122.

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 of the United States of America* **98**, 5922-5926.

Martin RC, Mok MC, Mok DWS. 1999a. A gene encoding the cytokinin enzyme zeatin O-xylosyltransferase of Phaseolus vulgaris. *Plant Physiology* **120**, 553-557.

Martin RC, Mok MC, Mok DWS. 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**, 284-289.

Martin RC, Mok MC, Shaw G, Mok DWS. 1989. An enzyme mediating the conversion of zeatin to dihydrozeatin in phaseolus embryos. *Plant Physiology* **90**, 1630-1635.

Mason MG, Li J, Mathews DE, Kieber JJ, Schaller GE. 2004. Type-B response regulators display overlapping expression patterns in Arabidopsis. *Plant Physiology* **135**, 927-937.

Mathevon C, Pierrel F, Oddou JL, Garcia-Serres R, Blonclin G, Latour JM, Menage S, Gambarelli S, Fontecave M, Atta M. 2007. TRNA-modifying MiaE protein from Salmonella typhimurium is a nonheme diiron monooxygenase. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 13295-13300.

Mauk CS, Langille AR. 1978. Physiology of Tuberization in *Solanum tuberosum* L: *cis*-Zeatin Riboside in the Potato Plant: Its Identification and Changes in Endogenous Levels as Influenced by Temperature and Photoperiod. *Plant Physiology* **62**, 438-442.

Mauk CS, Langille AR. 1978. Physiology of Tuberization in *Solanum tuberosum* L: *cis*-Zeatin Riboside in the Potato Plant: Its Identification and Changes in Endogenous Levels as Influenced by Temperature and Photoperiod. *Plant Physiology* **62**, 438-442.

Maxwell BB, Kieber JJ. 2004. Cytokinin signal transduction. *Plant hormones. Biosynthesis, signal transduction, action!*: Kluwer academic publishers, 321-349.

Mik V, Szuecova L, Smehilova M, Zatloukal M, Dolezal K, Nisler J, Gruz J, Galuszka P, Strnad M, Spichal L. 2011. N9-substituted derivatives of kinetin: Effective anti-senescence agents. *Phytochemistry* **72**, 821-831.

Miller CO, Skoog F, Okumura FS, Vonsaltza MH, Strong FM. 1956. Isolation, structure and synthesis of kinetin, a substance promoting cell division. *Journal of the American Chemical Society* **78**, 1375-1380.

Miller CO, Skoog F, Vonsaltza MH, Strong FM. 1955. Kinetin, a cell division factor from deoxyribonucleic acid. *Journal of the American Chemical Society* **77**, 1392-1392.

Miyata S, Urao T, Yamaguchi-Shinozaki K, Shinozaki K. 1998. Characterization of genes for two-component phosphorelay mediators with a single HPt domain in Arabidopsis thaliana. *Febs Letters* **437**, 11-14.

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. *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 of the United States of America* **103**, 16598-16603.

Moffatt BA, Wang L, Allen MS, Stevens YY, Qin WS, Snider J, von Schwartzenberg K. 2000. Adenosine kinase of arabidopsis. Kinetic properties and gene expression. *Plant Physiology* **124**, 1775-1785.

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

Morris RO. 1986. Genes specifying auxin and cytokinin biosynthesis in phytopathogens. *Annual Review of Plant Physiology and Plant Molecular Biology* **37**, 509-538.

Motyka V, Faiss M, Strnad M, Kaminek M, Schmulling T. 1996. Changes in cytokinin content and cytokinin oxidase activity in response to derepression of ipt gene transcription in transgenic tobacco calli and plants. *Plant Physiology* **112**, 1035-1043.

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.

Mougel C, Zhulin IB. 2001. CHASE: an extracellular sensing domain common to transmembrane receptors from prokaryotes, lower eukaryotes and plants. *Trends in Biochemical Sciences* **26**, 582-584.

Murashige T, Skoog F. 1962. A Revised Medium for Rapid Growth and Bio Assays 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.

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C. 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.

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.

Paces V, Werstiuk E, Hall RH. 1971. Conversion of N-(delta-isopentenyl)adenosine to adenosine by enzyme activity in tobacco tissue. *Plant Physiology* **48**, 775-778.

Parker CW, Badenoch-Jones J, Letham DS. 1989. 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.

Parker CW, Cowley DE, Letham DS, Macleod JK. 1972. Raphanatin, an unusual purine derivative and a metabolite of zeatin. *Biochemical and Biophysical Research Communications* **49**, 460-466.

Parker CW, Letham DS. 1973. Regulators of cell-division in plant tissues. 16. Metabolism of zeatin by radish cotyledons and hypocotyls. *Planta* **114**, 199-218.

Pernisova M, Klima P, Horak J, Valkova M, Malbeck J, Soucek P, Reichman P, Hoyerova K, Dubova J, Friml J, Zazimalova E, Hejatko J. 2009. Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 3609-3614.

Pernisova M, Klima P, Horak J, Valkova M, Malbeck J, Soucek P, Reichman P, Hoyerova K, Dubova J, Friml J, Zazimalova E, Hejatko J. 2009. Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 3609-3614.

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.

Persson BC, Esberg B, Olafsson O, Bjork GR. 1994. Synthesis and function of isopentenyl adenosine derivatives in tRNA. *Biochimie* **76**, 1152-1160.

Persson BC, Olafsson O, Lundgren HK, Hederstedt L, Bjork GR. 1998. The ms(2)io(6)A37 modification of tRNA in Salmonella typhimurium regulates growth on citric acid cycle intermediates. *Journal of Bacteriology* **180**, 3144-3151.

Pertry I, Václavíková K, Depuydt S, Galuszka P, Spíchal L, Temmerman W, Stes E, Schmülling T, Kakimoto T, Van Montagu MCE, Strnad M, Holsters M, Tarkowski P, Vereecke D. 2009. Identification of Rhodococcus fascians cytokinins and their modus operandi to reshape the plant. *Proceedings of the National Academy of Sciences of the United States of America* **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.

Punwani JA, Hutchison CE, Schaller GE, Kieber JJ. 2010. The subcellular distribution of the Arabidopsis histidine phosphotransfer proteins is independent of cytokinin signaling. *Plant Journal* **62**, 473-482.

Purvis AC, Barmore CR. 1981. Involvement of ethylene in chlorophyll degradation in peel of citrus fruits. *Plant Physiology* **68**, 854-856.

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.

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.

Riefler M, Werner T, Heyl A. 2007. Cytokinins. In: Roberts K, ed. *Handbook of plant science*, Vol. 1: Wiley, 369-376.

Richmond AE, Lang A. 1957. Effect of kinetin on the protein content and survival of detached *Xanthium* leaves. *Science* **125**, 650-651.

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.

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.

Rosenbaum N, Gefter ML. 1972. Delta 2 -isopentenylpyrophosphate: transfer ribonucleic acid 2 -isopentenyltransferase from Escherichia coli. Purification and properties of the enzyme. *Journal of Biological Chemistry* **247**, 5675-5680.

Ruzicka K, Simaskova M, Duclercq J, Petrasek J, Zazimalova E, Simon S, Friml J, Van Montagu MCE, Benkova E. 2009. Cytokinin regulates root meristem activity via modulation of the polar auxin transport. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 4284-4289.

Saenz L, Jones LH, Oropeza C, Vlacil D, Strnad M. 2003. Endogenous isoprenoid and aromatic cytokinins in different plant parts of Cocos nucifera (L.). *Plant Growth Regulation* **39**, 205-215.

Sakai H, Honma T, Aoyama T, Sato S, Kato T, Tabata S, Oka A. 2001. ARR1, a transcription factor for genes immediately responsive to cytokinins. *Science* **294**, 1519-1521.

Sakakibara H. 2004. Cytokinin biosynthesis and metabolism. In: Davies PJ, ed. *Plant hormones. Biosynthesis, signal transduction, action!*: Kluwer academic publishers, 95-114.

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

Sakamoto T, Sakakibara H, Kojima M, Yamamoto Y, Nagasaki H, Inukai Y, Sato Y, Matsuoka M. 2006. Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. *Plant Physiology* **142**, 54-62.

Shani E, Ben-Gera H, Shleizer-Burko S, Burko Y, Weiss D, Ori N. 2010. Cytokinin Regulates Compound Leaf Development in Tomato. *Plant Cell* **22**, 3206-3217.

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.

Schmulling T, Werner T, Riefler M, Krupkova E, Manns IBY. 2003. Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. *Journal of Plant Research* **116**, 241-252.

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.

Skoog F, Armstron.Dj, Cherayil JD, Hampel AE, Bock RM. 1966. Cytokinin activity: localization in transfer RNA preparations. *Science* **154**, 1354-1356.

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

Skoog F, Miller CO. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symposia of the Society for Experimental Biology* **11**, 118 - 130.

Skoog F, Strong FM, Miller CO. 1965. Cytokinins. *Science* **148**, 532-533.

Sondheimer E, Tzou DS. 1971. The Metabolism of Hormones during Seed Germination and Dormancy II. The Metabolism of 8-^{14}C-Zeatin in Bean Axes. *Plant Physiology* **47**, 516-520.

Song YH, Yoo CM, Hong AP, Kim SH, Jeong H, Shin SY, Kim HJ, Yun DJ, Lim CO, Bahk JD, Lee SY, Nagao RT, Key JL, Hong JC. 2008. DNA-binding study identifies C-box and hybrid C/G-box or C/A-box motifs as high-affinity binding sites for STF1 and LONG HYPOCOTYL5 proteins. *Plant Physiology* **146**, 1862-1877.

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.

Sramek F, Dubsky M. 2011. Occurrence and correction of lime-induced chlorosis in petunia plants. *Plant Soil and Environment* **57**, 180-185.

Stirk W, Václavíková K, Novák O, Gajdošová S, Kotland O, MOTYKA V, Strnad M, Van Staden J. submitted. Involvement of *cis*-zeatin, dihydrozeatin and aromatic cytokinins in germination and seedling establishment of maize, oats and lucerne. *Journal of Plant Growth Regulation*.

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.

Strnad M, Hanus J, Vanek T, Kaminek M, Ballantine JA, Fussell B, Hanke DE. 1997. Meta-topolin, a highly active aromatic cytokinin from poplar leaves (Populus x canadensis Moench, cv Robusta). *Phytochemistry* **45**, 213-218.

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

Sunderland, Wells B. 1968. Plastid structure and development in green callus tissue of *Oxalis dispar*. *Annals of Botany* **32**, 327-346.

Suzuki T, Imamura A, Ueguchi C, Mizuno T. 1998. Histidine-containing phosphotransfer (HPt) signal transducers implicated in His-to-Asp phosphorelay in Arabidopsis. *Plant and Cell Physiology* **39**, 1258-1268.

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizuno T. 2001. The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. *Plant and Cell Physiology* **42**, 107-113.

Suzuki T, Sakurai K, Imamura A, Nakamura A, Ueguchi C, Mizuno T. 2000. Compilation and characterization of histidine-containing phosphotransmitters implicated in His-to-Asp phosphorelay in plants: AHP signal transducers of Arabidopsis thaliana. *Bioscience Biotechnology and Biochemistry* **64**, 2486-2489.

Sweere U, Eichenberg K, Lohrmann J, Mira-Rodado V, Baurle I, Kudla J, Nagy F, Schafer E, Harter K. 2001. Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. *Science* **294**, 1108-1111.

Sykorova B, Kuresova G, Daskalova S, Trckova M, Hoyerova K, Raimanova I, Motyka V, Travnickova A, Elliott MC, Kaminek 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.

Šmehilová M, Galuszka P, Bilyeu KD, Jaworek P, Kowalska M, Šebela M, Sedláová M, English JT, Frébort I. 2009. Subcellular localization and biochemical comparison of cytosolic and secreted cytokinin dehydrogenase enzymes from maize. *Journal of Experimental Botany* **60**, 2701-2712.

Tajima Y, Imamura A, Kiba T, Amano Y, Yamashino T, Mizuno T. 2004. Comparative studies on the type-B response regulators revealing their distinctive properties in the His-to-Asp phosphorelay signal transduction of Arabidopsis thaliana. *Plant and Cell Physiology* **45**, 28-39.

Takei K, Sakakibara H, Sugiyama T. 2001. Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. *Journal of Biological Chemistry* **276**, 26405-26410.

Takei K, Yamaya T, Sakakibara H. 2004. Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-Zeatin. *Journal of Biological Chemistry* **279**, 41866-41872.

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 x canadensis leaves by LC-(+)ESI-MS and capillary liquid chromatography/frit-fast atom bombardment mass spectrometry. *Physiologia Plantarum* **117**, 579-590.

Taya Y, Tanaka Y, Nishimura S. 1978. 5'-AMP is a direct precursor of cytokinin in *Dictyostelium discoideum*. *Nature* **271**, 545-547.

To JPC, Haberer G, Ferreira FJ, Deruere J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ. 2004. Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* **16**, 658-671.

Turner JE, Mok DWS, Mok MC, Shaw G. 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**, 3714-3717.

Ueguchi C, Koizumi H, Suzuki T, Mizuno T. 2001a. Novel family of sensor histidine kinase genes in Arabidopsis thaliana. *Plant and Cell Physiology* **42**, 231-235.

Ueguchi C, Sato S, Kato T, Tabata S. 2001b. The AHK4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in Arabidopsis thaliana. *Plant and Cell Physiology* **42**, 751-755.

Upfold SJ, Vanstaden J. 1990. Cytokinins in cut carnation flowers. VII. The effect of zeatin and dihydrozeatin derivatives on flower longevity. *Plant Growth Regulation* **9**, 77-81.

Urbonavicius J, Qian O, Durand JMB, Hagervall TG, Bjork GR. 2001. Improvement of reading frame maintenance is a common function for several tRNA modifications. *Embo Journal* **20**, 4863-4873.

Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B, Novak O, Strnad M, Ludwig-Muller J, Oelmuller R. 2008. The role of auxins and cytokinins in the mutualistic interaction between *Arabidopsis* and *Piriformospora indica*. *Molecular Plant-Microbe Interactions* **21**, 1371-1383.

Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B, Novak O, Strnad M, Ludwig-Muller J, Oelmuller R. 2008. The role of auxins and cytokinins in the mutualistic interaction between Arabidopsis and Piriformospora indica. *Molecular Plant-Microbe Interactions* **21**, 1371-1383.

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.

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, Kollmer I, Bartrina I, Holst K, Schmulling T. 2006. New insights into the biology of cytokinin degradation. *Plant Biology* **8**, 371-381.

Yamada H, Koizumi N, Nakamichi N, Kiba T, Yamashino T, Mizuno T. 2004. Rapid response of Arabidopsis T87 cultured cells to cytokinin through His-to-Asp phosphorelay signal transduction. *Bioscience Biotechnology and Biochemistry* **68**, 1966-1976.

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T. 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.

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.

Yu YB, Yang SF. 1979. Auxin-induced ethylene production and its inhibition by aminoethyoxyvinylglycine and cobalt ion. *Plant Physiology* **64**, 1074-1077.

Zažímalová E, Kamínek M, Březinová A, Motyka V. 1999. Control of cytokinin biosynthesis and metabolism. In: Hooykaas P, Hall M, Libbenga K, eds. *Biochemistry and Molecular Biology of Plant Hormones*. Amsterdam: Elsevier Science B.V., 141-160.