Charles University

Faculty of Science

Study programme: Biochemistry

Branch of study: Biochemistry

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Characterization and significance of *N*-glucosyltransferase pathway as a tool for regulation of cytokinin homeostasis in plants

Charakterizace a význam *N*-glukosyltransferasové dráhy jako nástroje regulace homeostáze cytokininů v rostlinách

Diploma thesis

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Praha 2019

Prohlášení

Prohlašuji, že jsem tuto diplomovou práci vypracovala samostatně pod vedením školitele Ing. Václava Motyky, CSc. a všechny použité prameny jsem řádně citovala. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Statement

I hereby state that I have completed this thesis as an independent work and that I have properly cited all literature and other information sources I have used. Neither this thesis nor its parts have been submitted to achieve same or any other academic title.

V Praze / In Prague, _____

Lucie Doležálková

Acknowledgements

Firstly, I would like to express my deepest gratitude to my supervisor, Ing. Václav Motyka, CSc. for his patient guidance throughout this work. His long-term kind leadership introduced me to science and plant physiology. He kept his door opened for me whenever I ran into a trouble spot or had a question about my research or writing.

My special thanks belong to Ing. Eva Pokorná, Ph.D., who supported me both practically (in the laboratory and during writing) and mentally when results did not reflect the effort put into experiments. Also, I would like to acknowledge Marie Korecká, Bc. and Ing. Petre Ivanov Dobrev, CSc. who especially helped me with experimental work.

It is my pleasure to mention here prof. RNDr. Eva Zažímalová, CSc. who set a cornerstone of the bridge connecting department of Biochemistry at Charles University and Laboratory of Hormonal Regulations in Plants at the Institute of Experimental Botany CAS.

I am very grateful to my consultant doc. RNDr. Helena Ryšlavá, CSc. for her assistance in formal finalization of this thesis and her precious input on the Biochemistry departments' requirements.

Last, but not least, I'd like to thank my family and friends, who supported me continuously not only throughout my master studies. I can't thank enough to my partner for all of his comments, pushes and hugs. This accomplishment would not have been possible without them.

Abstrakt

Deriváty adeninu známé jako cytokininy (CK) jsou jednou ze základních skupin rostlinných hormonů (neboli fytohormonů) podílející se spolu s dalšími významnou měrou na řízení většiny fyziologických procesů. I přes bouřlivý rozvoj výzkumu fytohormonů nám šíře a komplexita metabolických a signalizačních drah spolu s jejich provázaností umožňuje odkrývat stále nová tajemství. *N*-glukosidy cytokininů (konkrétně *trans*-zeatin-7-glukosid, *tZ*7G, a *trans*-zeatin-9-glukosid, *tZ*9G) byly dlouhodobě považovány za ireverzibilně deaktivované produkty metabolismu aktivních forem (v tomto případě *trans*-zeatinu, *tZ*). Avšak senescenční biotesty na listech ovsa setého (*Avena sativa* cv. Abel) prokázaly aktivitu *tZ*9G. Toto zjištění může být vysvětleno možností metabolické přeměny na *O*-glukosidy. V této práci jsme se rozhodli otestovat aktivitu zmíněných glukosidů (*tZ*7G a *tZ*9G) na příbuzném modelu ovsa (*Avena sativa* cv. Patrik) a prozkoumat též metabolické konverze podobných látek – N^6-(Δ^2-isopentenyl)adeninu (iP) a jeho *N7*- a *N9*- glukosidů (iP7G a iP9G). Zatímco aktivitu *tZ*9G v senescenčních biotestech jsme potvrdili i pro model *Avena sativa* cv. Patrik, metabolické konverze na *O*-glukosidy ve významné míře pozorovány nebyly. Kromě vlivu zmíněných cytokininů na listové segmenty jsme se rozhodli prozkoumat vliv týchž látek na kořeny *Arabidopsis thaliana* (L.) Heynh, u kterých jsme kromě fenotypových projevů sledovali i změny genové exprese. I když se naše původní hypotéza nepotvrdila v plné šíři, výsledky získané na obou rostlinných modelech zpochybňují vžitou představu o CK *N*-glukosidech, přinejmenším glykosylovaných v *N9* poloze, jako neaktivních a ireversibilních produktech metabolismu CK a naznačují jejich vyšší významnost pro rostliny, než se dosud předpokládalo. (V angličtině)

Klíčová slova: cytokinin, glukosid, senescenční biotesty, kořenové biotesty

Abstract

Adenine derivatives called cytokinins (CK) are a group of plant hormones, which in cooperation with other plant hormones orchestrates almost every aspect of plant growth and development. Despite the rapid progress in plant hormone research, there are still many aspects we may shed light on due to the metabolic and signalling pathways redundancy and the network complexity with crosstalk hubs. CK *N*-glucosides (in this case *trans*-zeatin-7-glucoside, *tZ*7G, and *trans*-zeatin-9-glucoside, *tZ*9G) have been traditionally viewed as irreversibly deactivated products of CK active form metabolism (in this regard *trans*-zeatin, *tZ*). Nevertheless, the *tZ*9G antisenescent activity was shown in oat leaf senescence bioassays (*Avena sativa* cv. Abel) and the possibility of metabolic conversion to O-glucosides was hypothesized. The aim of this work was to test the hypothesis on the close model of oat (*Avena sativa* cv. Patrik) and to examine also the metabolic conversion of related substances – N^6-(Δ^2-isopentenyl)adenine (iP) and its *N7*- and *N9*- glucosides (iP7G and iP9G). While the senescence retardation caused by exogenous *tZ*9G application was confirmed in the *Avena sativa* cv. Patrik, metabolic conversions to *O*-glucosides remain to be verified. Besides the effects of above-mentioned substances on the oat leaf segments, we decided to examine their influence on phenotype and genes expression effects also on Arabidopsis thaliana (L.) Heynh roots. Although our previous hypothesis has not been fully confirmed, the obtained results on both monocot and dicot plant models question a general notion of CK *N*-glucosides, at least those glycosylated at *N9*-position, as inactive and irreversible CK forms and suggest their higher importance for plants than previously considered.

Keywords: cytokinin, glucoside, senescence bioassay, root bioassay

Contents

List of abbreviations

ABCG	ATP-binding cassette transporter G subfamily
AHK	cytokinin receptor ARABIDOPSIS HISTIDINE KINASE
AHP	ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER protein
AP2	transcription factor APETALA2
ARR	ARABIDOPSIS RESPONSE REGULATOR protein family
BA	N^6-benzyladenine
bp	base pair
CHASE	cyclases/histidine kinases associated sensory extracellular domain
cDNA	complementary DNA
CK	cytokinin
CKI1	histidine kinase CYTOKININ INDEPENDENT 1
CKX	cytokinin oxidase/dehydrogenase
CRE1	cytokinin receptor CYTOKININ RESPONSE 1
CRF	transcription factor family CYTOKININ RESPONSE FACTOR
CYP	cytochrome P450
cZ	*cis*-zeatin
*cZ*9G	*cis*-zeatin-9-glucoside
*cZ*OG	*cis*-zeatin-*O*-glucoside
*cZ*R	*cis*-zeatin riboside
*cZ*ROG	*cis*-zeatin riboside-*O*-glucoside
DAS	days after sowing
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
DZ	dihydrozeatin
DZ7G	dihydrozeatin-7-glucoside
DZ9G	dihydrozeatin-9-glucoside
DZOG	dihydrozeatin-*O*-glucoside
DZR	dihydrozeatin riboside
DZRMP	dihydrozeatin riboside monophosphate
DZROG	dihydrozeatin riboside-*O*-glucoside

ENT	EQUILIBRATIVE NUCLEOSIDE TRANSPORTER
ERF	transcription factor family ETHYLENE RESPONSIVE FACTOR
FW	fresh weight
HMBPP	*(E)*-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HPLC	high-performance liquid chromatography
iP	N^6-(Δ^2-isopentenyl)adenine
iP7G	N^6-(Δ^2-isopentenyl)adenine-7-glucoside
iP9G	N^6-(Δ^2-isopentenyl)adenine-9-glucoside
iPR	N^6-(Δ^2-isopentenyl)adenosine
iPRDP	N^6-(Δ^2-isopentenyl)adenosine diphosphate
iPRMP	N^6-(Δ^2-isopentenyl)adenosine monophosphate
iPRTP	N^6-(Δ^2-isopentenyl)adenosine triphosphate
IPT	isopentenyltransferase
Kin	kinetin
LOG	enzyme LONELY GUY
MCX	mixed-mode cation exchanger
MEP	methylerythritol
MS medium	Murashige and Skoog medium
MS salt	Murashige and Skoog Basal Salt Mixture
MVA	mevalonate
PCR	polymerase chain reaction
PUP	PURINE PERMEASE
qPCR	quantitative polymerase chain reaction (also real-time polymerase chain reaction)
RAM	root apical meristem
RT-PCR	reverse transcription polymerase chain reaction
SAM	shoot apical meristem
SD	standard deviation of the mean
SPE	solid phase extraction
TAE buffer	buffer solution containing Tris-acetic acid-EDTA
Tris	tris(hydroxymethyl)aminomethane

tZ	*trans*-zeatin
*tZ*7G	*trans*-zeatin-7-glucoside
*tZ*9G	*trans*-zeatin-9-glucoside
*tZ*OG	*trans*-zeatin-*O*-glucoside
*tZ*R	*trans*-zeatin riboside
*tZ*RDP	*trans*-zeatin riboside diphosphate
*tZ*RMP	*trans*-zeatin riboside monophosphate
*tZ*RTP	*trans*-zeatin riboside triphosphate
*tZ*ROG	*trans*-zeatin riboside-*O*-glucoside
UGT	uridine diphosphate-glycosyltransferase
wol	recessive mutation in cytokinin receptor *wooden leg*
ZRED	zeatin reductase

1 Introduction

The metabolism, growth, and development of multicellular organisms such as higher plants and animals are determined by the emission of signal compounds including hormones [1]. Commonly are plant hormones defined as a group of naturally occurring organic substances which influence physiological processes at low concentrations [2]. Despite this ambiguous definition and similar naming convention (plant hormones or phytohormones vs. animal hormones) due to the scientific research history, we should emphasize the key differences between these two groups. Whereas animal hormones are typically secreted by specialized glandular cells and emitted to distant cells, e.g. via the blood circulation, phytohormones are produced by different cell types within the plant body and affect wider range cells from neighbouring to distant (when transported by phloem or xylem) [1]. Going back to the Greek origin of the word hormone, meaning "to stimulate" or "to set in motion", it seems to fulfil rather a definition of plant hormone than an older concept used in animal physiology [2].

The existence of organ-forming substances (whose distribution leads to morphogenesis) was firstly postulated in 1880' by German botanist Julius von Sachs [3]. Differential origin of these substances was discussed – from proteins [4] to nutritional factors [5] or carbon:nitrogen ratio [6]. Nevertheless, it lasted nearly 40 years to prove the evidence of postulated substances [3]. The first person who explained the reaction of oat coleoptiles to the light by the flow of growth substance was Paál in 1919 [7]. This led to isolation [8] and later characterization [9] of the first discovered plant hormone – auxin. To these days, we distinguish several plant hormone groups including abscisic acid, auxins, brassinosteroids, cytokinins, ethylene, gibberellins, jasmonates, salicylic acid, strigolactones [10], and other hormones such as karrikins [11], nitric oxide [10], peptide hormones [12],

polyamines [13], and triacontanol [14]. Strikingly, most phytohormones are involved in a great number of physiological processes. This complexity allows integration of numeral internal and external stimuli into coordinated growth and development – the key to successful sessile life [10].

1.1 Cytokinins

The history of cytokinin research might be traced to 1941 when van Overbeek initiated the cell division in *Datura stramonium* explants grown on media enriched with auxin by addition of coconut milk [15] (previously reported efforts led to mitosis unaccompanied by cytokinesis [16]). The following research focused on other plant species (e.g. carrot – *Daucus carota* [17] or tobacco – *Nicotiana tabacum* [18]) and different active substance sources (coconut meat [18] or yeast extract [19]). The credit for isolation of the active substance went to Miller in 1955 when crystals of kinetin (named with respect to its ability to promote cytokinesis) were obtained from herring sperm autoclaved DNA [20]. Chemical structure of kinetin (Kin) was proposed as 6-furfurylaminopurine and confirmed by synthesis later in 1955 [21]. At the time of Kin discovery, generic name kinin for substances exhibiting the same type of biological activity was proposed. Nevertheless, confusion with animal physiology arose and name cytokinin (CK) was chosen for the respective group of plant hormones instead [22].

The early observation of CK physiological effect on plants regarded tissue cultures. Not only the acceleration of cell division was observed after Kin treatment, but also shoot formation could be initiated. Different developmental processes were triggered in unorganized plant cells depending on the CK/auxin concentration ratio in medium (shoot vs. root organogenesis, see Figure 1, p. 13) [23].

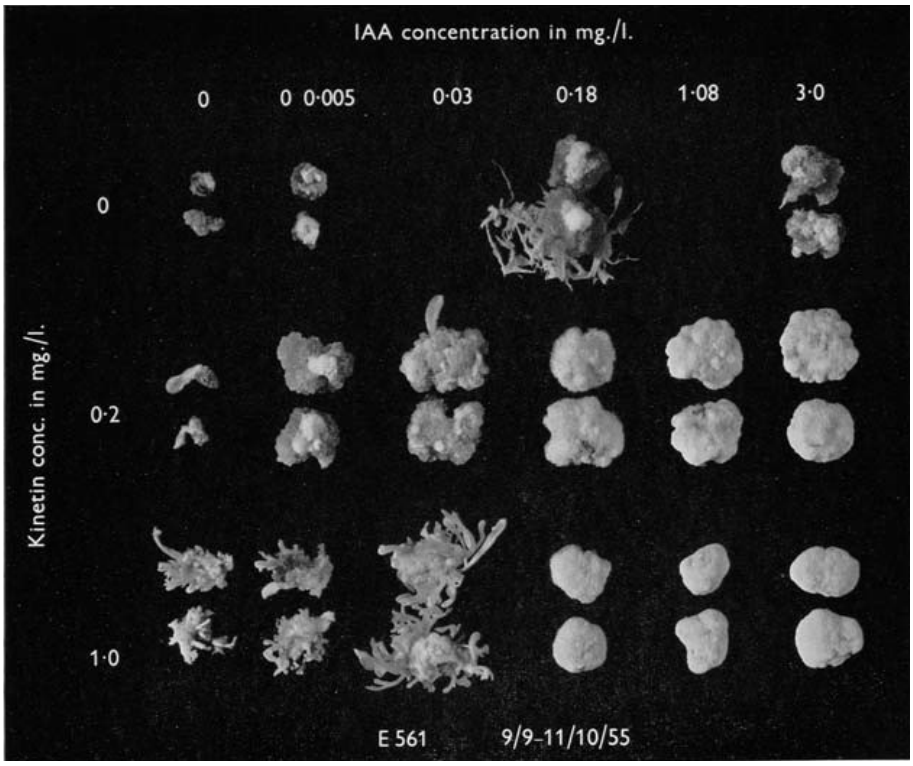


Figure 1: Effect of the auxin to cytokinin ratio on the pattern of development in tobacco explants (Nicotiana glauca x Nicotiana langsdorffiip hybrid)

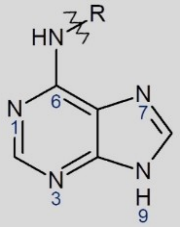
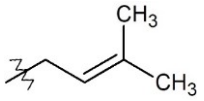
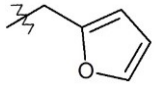
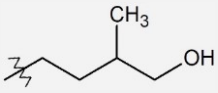
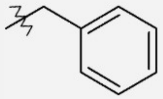
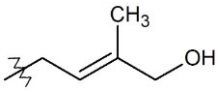
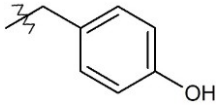
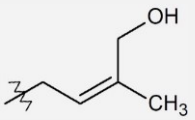
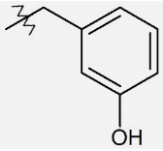
The horizontal axis shows auxin (indole-3-acetic acid) content in media (mg · L^{-1}), the vertical axis shows kinetin concentration respectively. High cytokinin levels lead to shoot organogenesis while auxin dominance causes root organogenesis. Development of callus tissue relies on balanced auxin to cytokinin content. Taken from [23].

Later, retardation of leaf senescence manifested by the preservation of chlorophyll and protein content was discovered [24] as well as shoot apical dominance suppression [25], seed germination [26] and others [27].

Concurrently with the studies of Kin effect on plants, various programs were initiated to learn structural requirements for CK activity. Furfuryl group of Kin was replaced with a wide variety of substituents, for instance, benzene (giving rise to N^6-benzyladenine), pyridyl, *n*-hexyl-, and *n*-amyl-. In most investigations, the intact purine ring appeared to be necessary for the activity [27], but the occurrence of such compounds in plants had not been

proved. Nevertheless, it was only due to the minute quantities of active substances in the plant material among the bulk of other structurally similar compounds [28].

Table 1: Chemical structures of representative cytokinins

Naturally occurring CKs are N^6-adenine derivatives, side-chain moieties (R) are shown below for the respective CKs 	
isoprene-derived side-chain	aromatic side-chain
N^6-(Δ^2-isopentenyl adenine) (iP) 	kinetin (Kin) 
dihydrozeatin (DZ) 	N^6-benzyladenine 
trans-zeatin (*tZ*) 	*meta*-topolin 
cis-zeatin (*cZ*) 	*ortho*-topolin 

First CK truly isolated from plant material was *trans*-zeatin (named after its source – maize, lat. *Zea mays, tZ*) in 1963. The procedure was as demanding as possibly extraction and fractionation of 70 kg of sweet maize kernels with yield 4.2 mg of the crystalline pure compound could have been [29]. The situation rapidly changed by the introduction of mass spectrometry to plant hormone research during the determination of *tZ* structure [30]. Following studies revealing novel substances in various plant species demonstrated that natural plant CKs are N^6-substituted adenines (see Table 1) with either an isoprene-derived side-chain (isoprenoid CKs) or aromatic side-chain (aromatic CKs). Even though first authors defined CKs based on their physiological activity, current naming relies on chemical structure rather than on results in bioassays. Indeed, the presence

of nucleosides, nucleotides, and sugar-conjugates imply extensive metabolic network for interconversions that should be integrated despite their lower activities. Contrary, the first CK representative – Kin, is currently perceived rather as plant growth regulator than endogenous plant hormone since its physiological presence *in planta* is questionable (typically, the plant growth regulators are chemical substances that have been developed for commercial reasons) [31].

As the research direction shifted from external application experiments to gene search, additional knowledge of CK biological function was obtained [32]. Original association with youth, growth and health (based on cell division stimulation and senescence retardation) turned to be premature when CK promoted cell differentiation [33] and even programmed cell death [34] were described. A recent view on CK function might be summarized as a trigger of a cellular change included in both developmental processes and adaptive responses to a changing abiotic and biotic environment [35] (see Figure 2, p. 16). The specificity of CK stimulus is dependent on active ligand location (CK metabolism and transport), signal timing (expression of signalling components) and state of recipient cell (cell history and crosstalk with other signals) [35].

1.1.1 Metabolism

CK metabolism might be divided into three basic steps: biosynthesis, (de)conjugation, and degradation. While there is only a little knowledge of the aromatic CKs origin, the light was shed on isoprenoid CKs biosynthesis (summarized in Figure 3, p. 23) [35]. Most of the knowledge was accumulated by *Arabidopsis thaliana* research (hereinafter referred to as Arabidopsis). Therefore, following sections will be focused primarily on processes and mechanisms observed in this model despite consequent limitations [36].

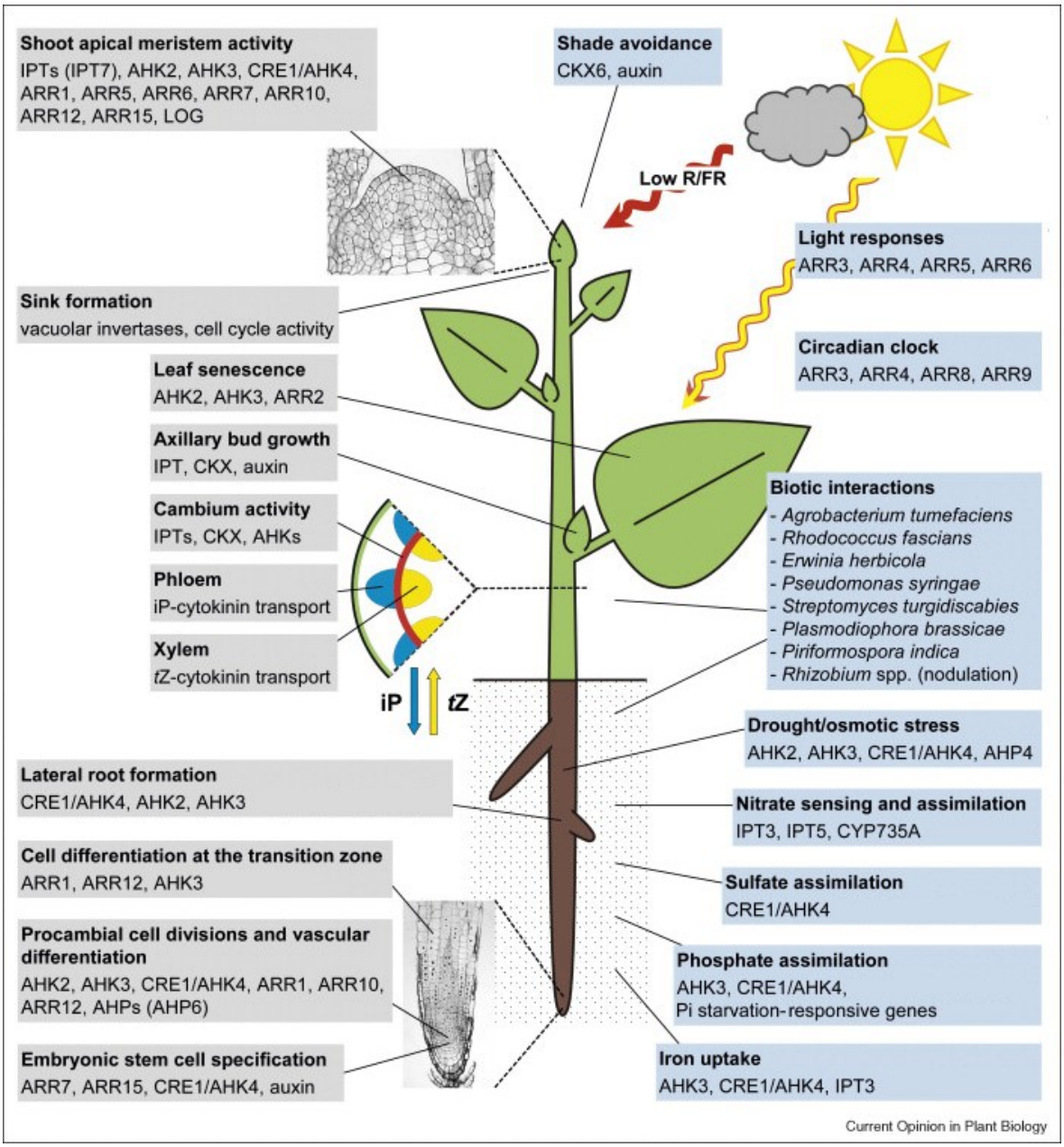


Figure 2: Developmental processes in Arabidopsis thaliana influenced by cytokinins.

The panels on the left show the processes regulated by cytokinin depending on intrinsic developmental programme while the right panels reflect environmental changes responses and biotic interactions regulated by cytokinins. Individual components of the metabolic and signalling machinery are listed: AHK - ARABIDOPSIS HISTIDINE KINASE, AHP - ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER, ARR - ARABIDOPSIS RESPONSE REGULATOR, CKX - cytokinin oxidase/dehydrogenase, CRE1 - CYTOKININ RESPONSE 1, CYP735A - cytochrome P450 enzyme, IPT - isopentenyltransferase, LOG - LONELY GUY.

Modified from [37].

Prevalent CKs in Arabidopsis are N^6-(Δ^2-isopentenyl adenine) (iP) and its hydroxylated *trans* isomer, *trans*-zeatin (*tZ*, for chemical structures see Table 1, p. 14). Other free CK bases are *cis*-zeatin (*cZ*) and dihydrozeatin (DZ) having a saturated side-chain. Additionally to the free bases, less active nucleotides, nucleosides and glycosides are present and even much more abundant. This allows a tight concentration control which prevents unregulated signalling [35].

The first dedicated step in the isoprenoid CK biosynthesis is catalysed by isopentenyltransferases (IPTs). Arabidopsis genome encodes 9 IPTs from two families with respect to adenine donor. The major family consists of 7 IPTs (IPT1 and IPT3-8) using preferably ATP and ADP as an isoprenoid acceptor [38]. In the rate-limiting reaction, the adenylate IPTs give rise to *tZ*-type and the majority of iP-type CKs [39]. The cellular localization of these proteins differs: IPT4 has been found in the cytoplasm, IPT7 is localized in mitochondria, IPT1, IPT5, and IPT8 occur in plastids. Localization of IPT3 depends on farnesylation, which directs the enzyme from plastids (in non-farnesylated form) to the nucleus or cytosol (when farnesylated) and regulates its activity [35]. Under physiological conditions, the second substrate for conjugation is dimethylallyl pyrophosphate (DMAPP) originated prevalently in plastids by methylerythritol (MEP) pathway or less importantly in cytosol by mevalonate (MVA) pathway [40]. Earlier suggested substrate, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), produced in MEP pathway was shown to be utilized only by bacterial IPTs (e.g. Tmr and Tzs from *Agrobacterium tumefaciens*) as well as an isoprenoid acceptor AMP [41]. Yielded CKs nucleotides depend on substrates combination, the most prevalent are N^6-(Δ^2-isopentenyl)adenosine triphosphate (iPRTP) and N^6-(Δ^2-isopentenyl)adenosine diphosphate (iPRDP). Under certain conditions, also N^6-(Δ^2-isopentenyl)adenosine

monophosphate (iPRMP), *trans*-zeatin riboside triphosphate (*tZ*RTP), *trans*-zeatin riboside diphosphate (*tZ*RDP), and *trans*-zeatin riboside monophosphate (*tZ*RMP) might be synthesized [35]. The prevalent products of this biosynthetic pathway, iP ribotides, might be subsequently converted to *tZ*-type CKs via the stereo-specific isoprenoid side-chain hydroxylation by the cytochrome P450 enzymes CYP735A1 and CYP735A2 [42]. Even though both enzymes are expressed predominantly in roots, the double mutants *cyp735a1 cyp735a2* show significant alternations in growth and development of aerial parts (retardation of shoot growth represented by reduction in rosette and shoot apical meristem i.e. SAM diameter, delayed abaxial trichome production, and enhanced shoot branching). Grafting experiments and exogenous CK application on the double mutants demonstrated the role of *tZ* long-distance signalling effect on shoot development [43] (see chapter 1.1.3 Transport, p. 28).

The *tZ*-type CKs might be converted to DZ-type CKs by zeatin reductase (ZRED) [44]. Up to date, this enzyme was found only in excised bean axes [45], bean embryos [46], and pea leaves [47]. Mediated isoprenoid side-chain saturation requires NADPH (but no NADH or ATP) [46] and possibly also a metal cofactor [47]. Despite its partial characterization, the ZRED role remains rather unclear [47]. Yielded DZ-type CKs show high activity in some bioassays and are not irreversibly degraded by cytokinin oxidase/dehydrogenase (CKX, for further details, see below) [46]. This, together with predominant occurrence in storage organs, suggests a specific role of DZ-type CKs as a source of bioactive CKs before the acceleration of *de novo* biosynthesis after germination [39].

The second group of IPTs (in Arabidopsis represented by IPT2 and IPT9) is entirely responsible for the formation of *cZ*-type and partially also for iP-type CKs by the tRNA

isopentenylation [38]. Interestingly, prenylated tRNA exists in all eukaryotic and prokaryotic organisms except for Archae [39]. This modification occurring on adenine at position 37 specific (UNN-)tRNAs (at the 3' adjacent to the anticodon with adenine on the third position) influences translational efficiency and fidelity, improves translation proofreading by decreasing misreading at the first position of the codon. When the third anticodon of a tRNA is adenine, the weak bond to the first codon uracil in mRNA might be strengthened by isopentenylation of adjacent adenine in tRNA [48]. The tRNA prenylation seems to play an important role under specific conditions (such as e.g. stress), yet it is not clear, to which content functions of prenylated tRNA might be associated with those of free CKs in plants [49]. Unlike the adenylate IPTs, the tRNA IPTs are expressed uniformly throughout the plant ontogenesis (independently of stimuli or changes in CK content) [50]. Therefore, the rate-limiting step of *cZ* biosynthesis is a degradation of isoprenylated tRNAs catalysed by ribonucleases in not fully elucidated manner [51]. Since double mutants *atipt2 9* lacking *cZ*-type CKs show no gross morphological changes, their functional relevance was questioned in Arabidopsis [38]. Later, root meristematic region reduction and related *cZ* role in protoxylem differentiation were described [52]. Moreover, *cZ* is a predominant CK in many plant species (e.g. liverwort *Conocephalum conicum*, moss *Plagiomnium undulatum*, fern *Pteridium aquilinum*, conifer *Pinus sylvestris*, monocots *Colchicum autumnale*, *Avena sativa,* and *Zea mays*, dicots *Nicotiana tabacum*, and *Plantago major*) [53] and alternative biosynthetic pathways are still under consideration [54].

The CK nucleotides resulting from IPTs' action undergo enzymatic dephosphorylation shared with the purine metabolic pathway (i.e. salvage pathway). The purine salvage pathway includes isoenzymes with broad substrate specificities, enabling them to act on CKs as well as on adenine [55].

A crucial role in the release of CK free bases is played by the LONELY GUY (LOG) protein family, identified firstly by a rice genetic screen for defects in the maintenance of shoot meristems [56]. In Arabidopsis, nine *LOG* genes were predicted as rice homologs (*LOG1-9*) while only seven proteins showed the phosphoribohydrolase activity (LOG1-5, LOG7 and LOG8) [57]. So-called one-step CK activation pathway mediated by LOGs directly converts CK nucleotides (iPRMP and *tZ*RMP) to free base forms with the release of a ribose 5'-monophosphate [56]. Historically postulated two-step activation pathway (where the CK riboside 5'-monophosphate is successively converted to free base by a nucleotidase [58] and a nucleosidase [59]) was shown to be only of minor importance [60], even despite the respective nucleosidase identification [61].

The levels of free bases can be decreased by CK conjugation with sugar moieties (mostly glucose) or through irreversible degradation by cytokinin dehydrogenase/oxidase (CKX) [62].

Two types of glucosylation have been distinguished – *N*-glucosylation occurring on the purine ring (at positions *N3-*, *N7-*, and *N9-*) and *O*-glucosylation occurring on the hydroxyl group of the side-chains of *tZ*, DZ, and *cZ* [55]. Neither *N*- or *O*- glucosides are able to trigger CK response through receptors [63] and are not degraded by CKX [64]. Whereas *N*-glucosides are perceived as terminal products of the irreversible deactivation [35], *O*-glucosides were shown to be β-glucosidase substrates and therefore are thought to serve rather as inactive storage forms [65].

Glycosylation by uridine diphosphate-glycosyltransferases (UGTs) is required in a number of biological processes, not only across the plant kingdom. The Arabidopsis CK-specific

UGTs are UGT76C1, UGT76C2 (both catalyse *N7*- and *N9*-glucosylation), and UGT85A1 (catalysing *O*-glucosylation) [35].

The *in vitro* studies show similar catalytic activities of UGT76C1 and UGT76C2 toward the tested CKs [66]. *UGT76C2* is steadily expressed throughout the developmental stages in contrast to *UGT76C1* exhibiting varying expression. Exogenous CK application leads to an increase in *UGT76C2* expression, but no modulation was shown for *UGT76C1* [67]. Both enzymes are localized in the cytosol and their physiological role is similar but to a different extent [68]. Modified phenotype under standard growth condition was observed only in *ugt76c2* mutant and manifested by smaller seeds [69]. Contrary, the *N*-glucosylation pathway seems to be important under stress conditions [70].

Whereas the *N*-glucosides are accumulated during the plant life (which correlates with their presumption of terminal metabolites), the *O*-glucosylation occurs specifically in senescent leaves which agrees with *UGT85A1* strongly upregulated expression [67].

The enzymatic CK degradation has been known since 1971 [71]; however, it took 30 years to reveal the dehydrogenation mechanism of the reaction [72]. Older classification of the enzyme as cytokinin oxidase is still in use [73-75] even though the term cytokinin dehydrogenase is more appropriate [72]. The flavoprotein cytokinin dehydrogenase/oxidase (CKX) mediates a cleavage the CK unsaturated N^6-side chain to produce a corresponding aldehyde and adenine (thus, DZ is resistant to the CKX activity) [76]. Molecular masses of plant CKX apoproteins calculated from gene sequences are rather uniform [77] in contrast to the reported molecular weights of plant enzyme extracts [44]. These differences might be partially explained by posttranslational modification – CKX

glycosylation was shown to play a role in pH optima, enzyme localization and its activity [77].

Arabidopsis genome analysis identified seven CKX genes (*CKX1-7*). The individual protein isoforms differ in their subcellular localization, substrate specificity and temporal pattern of expression. CKX1 and CKX3 are located in vacuole, CKX7 in cytosol and the other isoforms are transported to apoplast [52, 78].

1.1.2 Signalling

It took over 40 years to reveal the molecular mechanism of CK action. Initial studies based on CK exogenous application turned against researchers as they did not involve the use of plants with altered CK levels responding to them [32]. The first steps towards the elucidation of CK perception pathway were taken by Kakimoto. He successfully isolated the Arabidopsis mutant exhibiting CK responses independent on external CK application. In the screen of activation tagging, the gene *CYTOKININ INDEPENDENT 1* (*CKI1*) was identified and sequence analysis predicted homology to histidine kinase of bacterial two-component systems [79]. Although further investigations declined CKI1 role of CK receptor [80], the first hint about CK perception and signalling was utilized independently by three research groups to the discovery of authentic CK receptor CRE1/AHK4 [81, 82]. *CRE1* (*CYTOKININ RESPONSE 1*) was described by Arabidopsis mutant screen [81]. On the other hand, *AHK4* (*ARABIDOPSIS HISTIDINE KINASE 4*) was identified in similarity search with genome sequences [83] and the protein function was demonstrated later by targeted mutagenesis [82, 84]. Moreover, *CRE1/AHK4* is allelic to *wol* (*wooden leg*), a recessive mutation resulting in an exclusive xylem differentiation within the vascular tissues, characterized earlier [85] (*CRE1/AHK4 /WOL* hereinafter referred to as *AHK4*).

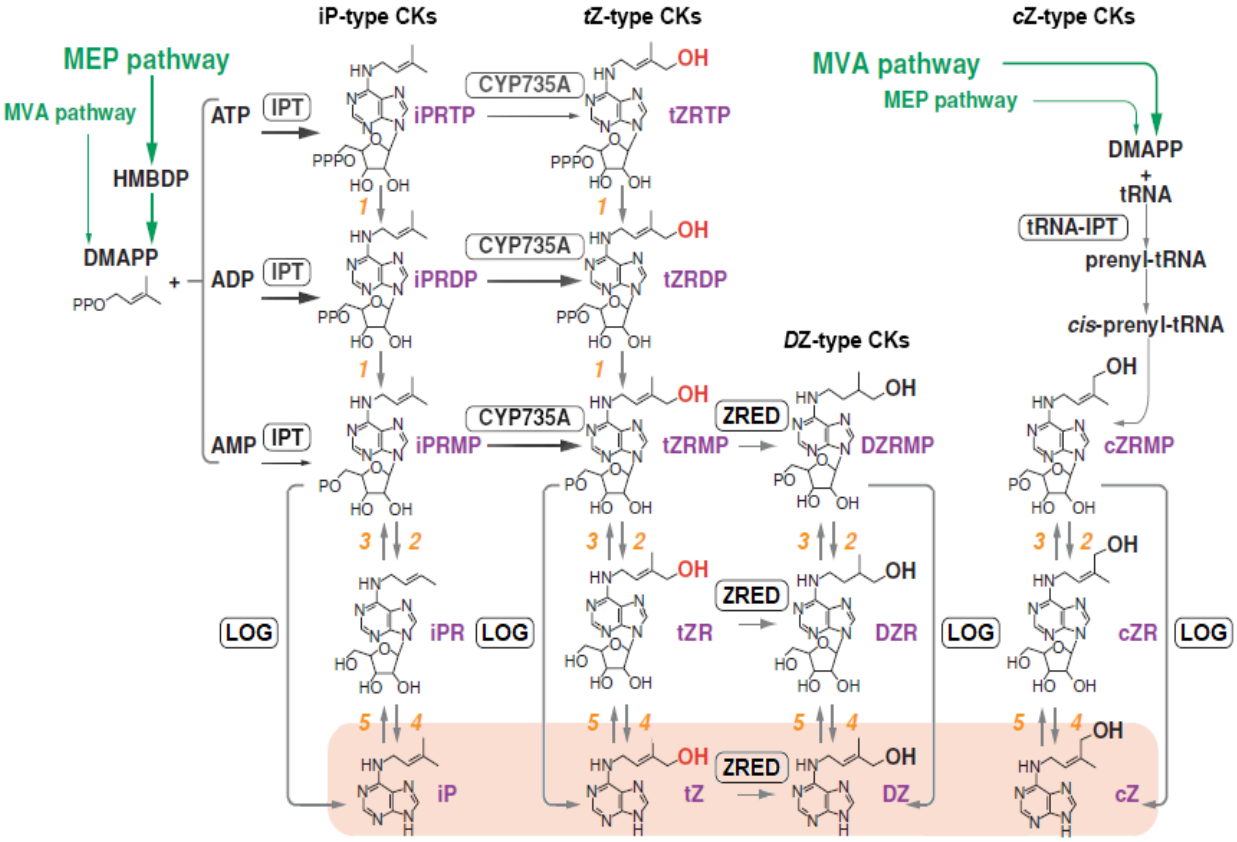


Figure 3: Isoprenoid cytokinin (CK) biosynthesis

First step in isoprenoid CK biosynthesis is catalysed by ISOPENTENYLTRANSFERASEs (IPTs) which conjugate the isoprenoid side-chains to adenine, preferably in form of ADP and ATP. The origin of isoprenoid precursors giving arise to isopentenyl adenine (iP) and trans-zeatin (tZ) might be traced to the methylerythritol phosphate (MEP) pathway, whereas a large fraction of the cis-zeatin (cZ) side-chain is derived from the mevalonate (MVA) pathway. Dephosphorylation of CK nucleotides is mediated by phosphatase (1), and release of free bases by nucleotidase (2), in the opposite direction nucleosides phosphorylation is catalysed by adenosine kinase (3). CK nucleosides might be converted to free bases by nucleosidase (4) and vice versa by purine nucleoside phosphorylase (5). These flows are not well characterized, the prospective enzymes are presumably part of the purine salvage pathway. Direct release of free bases from nucleotide monophosphates is mediated by phosphoribohydrolase family called Lonely guy (LOG). The iP-type CKs are converted into the corresponding tZ-type by CYP735A; tZ-type CKs might be subsequently hydrogenated to dihydrozeatin-types (DZ) by zeatin reductase (ZRED). The width of the arrowheads and lines indicates the strength of metabolic flow. Modified from [56].

Besides AHK4, also AHK2 and AHK3 were proposed by the genetic screening mentioned above as CK receptors [83] and later confirmed [86]. The Arabidopsis AHK family shows a high degree of sequence identity and a considerable degree of functional redundancy (loss-of-function mutations of single receptor displayed rather a subtle effect on phenotype) [87]. The *AHK4* is predominantly expressed in root, while both *AHK2* and *AHK3* show greater expression in the aerial parts [86]. Analysis of the ligand binding properties showed differences in CK affinities to AHK family members [87]. AHK2 resembled more closely to the AHK4 (notably AHK2 *in planta* required higher CK concentration) [88] – similar binding properties for both *tZ* and iP were found, while AHK3 strongly prefers *tZ* (probably as a response to a long-distance signal from roots) [89]. The crystal structure of AHK4 sensor domain shed light on the mechanism of iP discrimination and explained low activity of some CK representatives (*cZ*, *O*-glucosides and *N*-glucosides) [90]. Cellular localization of AHKs includes plasma membrane and endoplasmic reticulum membrane (for an overview see Figure 4, p. 29). Historically, only the receptors with extracellular binding domain were considered active (due to limited knowledge of CK transporters and analogy to the bacterial two-component system) [91]. Studies of AHKs binding properties showed a receptor inhibition at low pH level both in heterologous [89] and plant assays (e.g. the pH 5 led to irreversible AHK3 inactivation) [92]. With respect to the apoplast acidity (pH range 4.5 – 5.5), additional experiments revealed an important role of intracellular located receptors in CK signal transmission [93].

The membrane-spanning AHKs bind CKs to the CHASE (cyclases/histidine kinases associated sensory extracellular) domain at N-terminal ends. Elicited conformational receptor change triggers AHK dimer transphosphorylation on conserved His residue in the transmitter domain. The phosphoryl group is subsequently transferred to a conserved

Asp of the receiver domain at the C-terminal end and later to His in ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER (AHP) protein. Neither AHK2 nor AHK3 harbour intrinsic phosphatase activity of AHK4 [35]. The balance between interacting AHPs (de)phosphorylation depends on a ligand threshold concentration and represents protection from spurious activation [94].

Six AHPs are encoded by Arabidopsis genome (AHP1-6) [35]. Five of them (AHP1-5) shuttle between the cytosol and the nucleus to transfer the phosphoryl group to transcription factors regardless of CK concentration [95]. AHP6, also called pseudo-AHP, carries an inert Asn instead of necessary His [96]. Being unable to transfer phosphorylate group, AHP6 competes with AHP1-5 for interaction with AHKs and attenuates signalling. Restricted AHP6 expression enables to sharp the signalling output domains in specific processes and to define cell differentiation boundaries (during protoxylem differentiation [96] or phyllotaxis [97]).

First targets of AHP phosphorylation activity were revealed by several independent research groups in 1998 [98-100]. The respective proteins belong to ARABIDOPSIS RESPONSE REGULATOR (ARR) family which fall into four classes based on phylogenetic analysis and domain structure: type-A ARRs, type-B ARRs, type-C ARRs, and the ARABIDOPSIS PSEUDORESPONSE REGULATOR (APRRs) [101]. Out of numerous ARRs, only type-A and type-B ARRs directly mediate the CK responses (type-C ARRs might serve as modulators of CK signalling but their role is less characterized) [35]. Both type-A and type-B ARRs contain N-terminal phosphoryl receiver domain necessary for AHP interaction (see Figure 4, p. 29). Whereas 11 type-B ARRs (ARR1-2, ARR10-14, and ARR18-21) have also a Myb-related DNA binding domain, only a short C-terminal extension beyond the receiver domain is present in 10 type-A ARRs (ARR3-9,

and ARR15-17). Thus, type-B ARRs act as transcriptional activators of a subset of CK-regulated genes including type-A *ARR*s, which in return attenuate CK signalling and establish a negative feedback loop [102]. Functional redundancy described earlier at lower steps of CK signalling cascade escalate at ARR level [103, 104]. Only higher-order mutants show severe developmental defects and the dazzling network complexity with numerous modes of crosstalk complicate data interpretation [91].

Upon type-B ARR phosphorylation, receiver domain autoinhibition is relieved and conformational change triggers binding of the transcription factor to target sequences. Consistently with this model, B-type ARRs are present in nucleus independent of CK [105]. Signalling inactivation is ensured by intrinsic phosphatase activity of type-B receiver domain (together with type-A ARR competition of phosphorylated AHPs) and targeted protein degradation. The family of F-box proteins KISS ME DEADLY physically interacts with type-B ARRs and allows ubiquitin-mediated proteolysis. KISS ME DEADLY is the specificity component within S-PHASE KINASE-ASSOCIATED PROTEIN1/Cullin/F-box protein E3 ubiquitin ligase complex. By phosphorylated ARR type-B ubiquitination, the longevity of activated protein and transcriptional output are controlled [106]. A question regarding alteration of CK regulated proteolytic control within the type-B ARRs still remains unanswered [107].

Out of all type-B ARRs, genetic analysis indicates that ARR1, ARR10, and ARR12 play the most substantial roles in the regulation of transcriptional and physiological responses [108, 109]. Recent studies indicated 4 004 [110] up to approximately 10 000 (upon 10 days of CK induction) [111] candidate gene targets in Arabidopsis genome by immunoprecipitation sequencing. Not only the CK autoregulative genes (type-A ARRs, AHPs, AHKs and CKXs showed largely upregulation) were differentially expressed,

but also multiple negative regulators in auxin, abscisic acid, and salicylic acid pathways. In addition, *WUSCHEL* stimulation (required for SAM function and activity) should be mentioned [111].

The pleiotropic reaction to CKs is achieved through context-dependent transcriptional regulatory mechanisms such as chromatin accessibility. The recent study demonstrated overlaps between CK induced differentially expressed genes and differences in chromatin landscape. The type-B ARRs role in the CK regulated chromatin remodelling was proved, however, the mechanism and linked complexes should be further studied [74].

Besides competition of phosphorylated AHPs, type-A ARRs phospho-dependently interact with target proteins [101] (e.g. auxin efflux carriers in roots [112]). It has been shown that some of type-A ARRs are transcriptionally more responsive to different inputs than type-B ARRs which might modulate CK response in a broader context (auxin signalling [113], red light signalling [114], circadian clock oscillation [115] and others). The negative type-A ARRs regulation seems to be mediated by proteasomal degradation, however, the details have not been elucidated yet [116].

In parallel to the type-B ARRs act CYTOKININ RESPONSE FACTORs (CRFs), a subset of large AP2/ERF transcription factor family [35]. This protein group is defined by the presence of AP2 DNA binding domain (68 amino acids described first in APETALA2 or AP2). While the AP2 (playing a role in floral development) possess two AP2 domains, members of ERF-like family (including CRF) contain only one AP2 domain. ERF or ETHYLENE RESPONSIVE FACTOR mediates responses to the plant hormone ethylene and represents a group of stress response-related transcription factors [117]. *CRF2* and *CRF5* transcripts have been identified as transcriptionally rapidly upregulated

ones in response to CKs and named analogically to the related *ERF*. In contrast, the *CRF1*, *CRF3*, and *CRF4* transcription show minimal response to CKs. The last of the six-member family, *CRF6*, is also induced by CKs, but more steadily than *CRF2* and *CRF5* [118]. The CRFs were shown to interact with AHPs [119] independently on their phosphorylation state [117] and to form homo/heterodimers [119] to regulate gene transcription in a similar way as the type-B ARRs [118]. Even though the gene sets influenced by type-B ARRs and CRFs mostly overlap, the disruption of CRFs does not affect the CK induction of the type-A ARRs [117].

1.1.3 Transport

During years of CK research, important knowledge has been built regarding their biology. Strikingly, one aspect has been lagging – CK transport [32]. In early research, the role of root-sourced CKs in shoot growth and development was postulated. The xylem exudate truly contains CKs and their concentration varies in response to perturbations in the root environment. Exogenous CK application might overcome inhibition of the shoot growth associated with reduction of endogenous CK contents in xylem sap, but the mechanisms and causality remained to be elucidated (as well as a relationship to the proposed phloem mediated CK transport) [120]. Later analyses showed the biased distribution of *tZ*-type CKs in xylem sap and iP- and *cZ*-type CKs in phloem [121]. This compartmentalization allows recognition of a signal direction. Furthermore, differential expression of metabolic and signalling components and their sensitivity to CK substances should be considered (e.g. CYP735A, enzyme responsible for production of a majority of *tZ*-type CKs, is expressed predominantly in roots but a lack of the protein disturbs shoot growth; AHK3, preferably sensing the root-borne *tZ*-type CKs, is expressed in shoots) [122]. Nevertheless, the presence of molecular transporters is a prerequisite for long-distance transport.

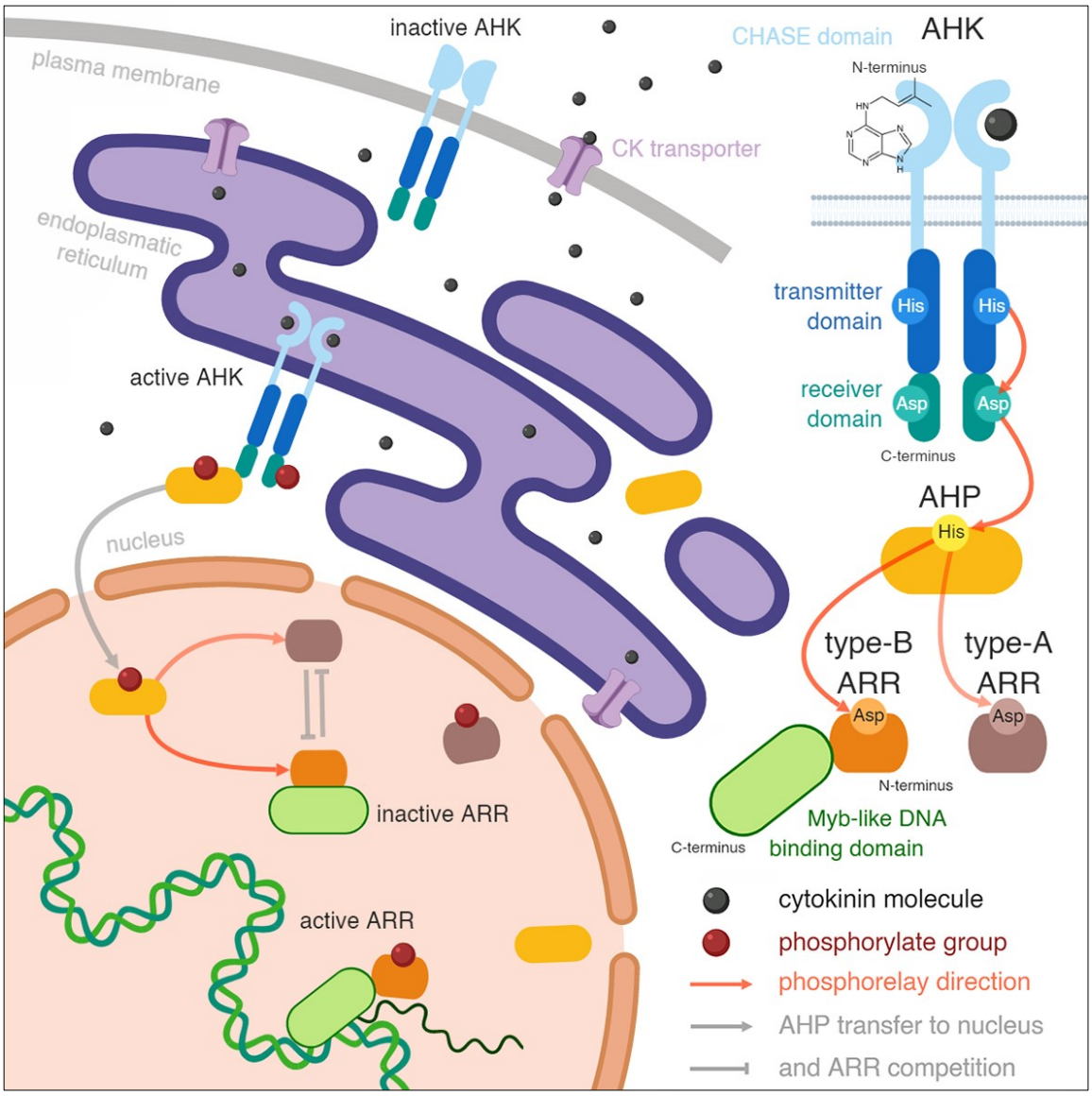


Figure 4: Schematic model of cytokinin (CK) perception pathway

The cellular localization of CK signalling machinery is illustrated at the left side, while the components and phosphotransfer steps are listed at the right part.

Upon CK binding, ARABIDOPSIS HISTIDINE KINASE (AHK) launches a phosphorelay signalling cascade. ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER (AHP) transmits the signal to the nucleus where two types of ARABIDOPSIS RESPONSE REGULATORs (ARRs) compete for the phosphoryl group. Only type-B ARRs act as transcription factors, since type-A ARRs lack the Myb-like DNA binding domain (thus, attenuate signalling). Upon phosphorylation, type-B ARR changes its conformation and allow DNA binding. Subcellular compartmentation plays an important role also in signal reception – endoplasmic reticulum-located AHKs are more active than those located to plasma membrane due to the lower extracellular pH level. Created with [123].

Up to date, three groups of CK transporters have been reported – PURINE PERMEASEs (PUPs), EQUILIBRATIVE NUCLEOSIDE TRANSPORTERs (ENTs), and G subfamily ATP-binding cassette (ABCG) transporters [32].

The first CK transporters were identified by complementation of a yeast mutant deficient in adenine uptake with an Arabidopsis cDNA expression library. Revealed *PUP1* belongs to a large gene family (*PUP1* – *PUP21* [124]) coding small, highly hydrophobic membrane proteins. The transporter capacity for adenine analogues in competition assays indicated CKs as transported substrates (for PUP1 particularly Kin and *tZ*) [125], which was later confirmed [126]. PUPs mediate a cellular CK proton-coupled import and are candidates for CK retrieval from xylem sap and phloem loading [124]. Recently, the developmental role of PUP14 was suggested. The cellular import was shown to decrease a CK response in the prospective cells [127]. This finding is in contradiction to previously mentioned inactivity of extracellular CHASE domains of AHKs [93]. Alternatively, ER receptors localization might serve as a reservoir capable of plasma membrane recruitment or an independent mechanism interrupting CK signalization [128]. Irrespective of the way, further investigation is necessary to unravel this enigma.

Unlike the PUP family demonstrated to transport mainly free bases, the ENTs action is limited to nucleoside CKs (notably ribosides iPR and *tZ*R) [129]. Arabidopsis ENT family consists of eight members: *ENT1-8*. Contrary to the naming convention (established by mammalian homologs), the seven members of Arabidopsis ENT family (ENT1-6, and ENT8) mediate a substrate import based on proton symport (not equilibrative transport along the concentration gradient) [124]. The only CK exporter known so far is ABCG14 involved in xylem loading (*abcg14* mutants show a 90% decrease of CK content in xylem sap) [130]. The ABCG family includes 43 proteins that participate for example

in the transport of abscisic acid, auxin, cutin, and wax [131]. Due to the undetermined biological and biochemical properties on some of them, there might be another ABCG CK transporter but ABCG14 [32].

With the molecular knowledge of CK transporters, the hypothesis of long-distance xylem transport was elegantly confirmed by a series of grafting experiments using *abcg14*, several *ipt* and *log* mutants and wild-type plants [132]. Nevertheless, the outlined phloem transport waits for more pieces to put together the puzzle of bi-directional vascular CK transport. While there is strong evidence of phloem CK transport role in vascular bundle development [133], little is known of the molecular principles of CK phloem loading [32].

Despite the mounting knowledge of CK transporters, the intracellular transport system is also largely unknown. Localization of CK metabolic machinery includes plastids (early steps of CK biosynthesis), cytosol (further processes), and vacuole (where several CK degradation enzymes reside), but prospective transporters have not been found [32].

2 Aims of the study

Previous studies have shown different physiological effects of exogenously applied *N*-glucosides of *trans*-zeatin (*tZ*) type-CKs and metabolic conversion of *tZ N9*-glucoside (*tZ*9G) but not *tZ N7*-glucoside (*tZ*7G) to *cZ O*-glucoside (*cZ*OG) and, in lesser extent also *tZ O*-glucoside (*tZ*OG) in oat (*Avena sativa* L. cv. Abel) [134]. The objective of this study was to examine metabolization of *N7*- and *N9*- CK glucosides, depending on availability of hydroxyl group for *O*-glucosylation, in another oat cultivar (*Avena sativa* L. cv. Patrik). For these experiments, six CK substances were tested: *tZ* and its *N7*- and *N9*- glucosides, N^6-(Δ^2-isopentenyl adenine) (iP) and its *N7*- and *N9*- CK glucosides.

Furthermore, investigation of a different plant model, *Arabidopsis thaliana* (L.) Heynh, was performed. In this system, physiological effects of exogenously applied *tZ*, iP and their *N7*- and *N9*-glucosides on the root growth inhibition were measured as well as the transcription levels of selected genes of CKs metabolism, signalling, and transport.

3 Material and methods

3.1 Material

3.1.1 Biological material

Seeds of oats (*Avena sativa* cv. Patrik) Selgen, Czech Republic

Seeds of *Arabidopsis thaliana* ecotype Columbia

provided by Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany of the Czech Academy of Sciences (Ing. Václav Motyka, CSc.)

3.1.2 Chemicals

Ambion, USA:

DNA-free Kit

Bioferm, Czech Republic:

ethanol

Olchemim, Czech Republic:

Cytokinin standards: *tZ*, *cZ*, *tZ*7G, *tZ*9G, iP, iP7G, iP9G, $^{2}H_{5}$-*tZ*, $^{2}H_{5}$-*tZ*R, $^{2}H_{5}$-*tZ*7G, $^{2}H_{5}$-*tZ*9G, $^{2}H_{5}$-*tZ*OG, $^{2}H_{5}$-*tZ*ROG, $^{2}H_{5}$-*tZ*RMP, $^{2}H_{3}$-DHZ, $^{2}H_{3}$-DHZR, $^{2}H_{3}$-DHZ9G, $^{2}H_{6}$-iP, $^{2}H_{6}$-iPR, $^{2}H_{6}$-iP7G, $^{2}H_{6}$-iP9G, $^{2}H_{6}$-iPRMP

Lach-Ner, Czech Republic:

ammonium hydroxide (solution 25% for liquid chromatography-mass spectrometry), calcium nitrate (tetrahydrate), ferric chloride hexahydrate, formic acid, hydrochloric acid, magnesium sulphate heptahydrate, methanol, potassium dihydrogen phosphate, sucrose

Lachema, Czech Republic:

potassium chloride, potassium hydroxide, sodium hydroxide

Linde, Germany:

liquid nitrogen

Lonza, Switzerland:

SeaKem LE Agarose

Promega, USA:

GoTaq qPCR Master Mix, M-MLV Reverse Transcriptase, PCR Nucleotide Mix, Recombinant RNasin Ribonuclease Inhibitor

Qiagen, Germany:

RNeasy Plant Mini Kit (50)

Sigma-Aldrich, USA:

agar, dimethyl sulfoxide (DMSO), 2-mercaptoethanol, Murashige and Skoog Basal Salt Mixture (MS salt), Tris Acetate-EDTA buffer (TAE buffer), tris(hydroxymethyl)aminomethane (Tris), Tween 20

Thermo Fisher Scientific, USA:

acetic acid (HPLC purity), acetonitrile (HPLC purity), DNA-free DNA Removal Kit, DNA Gel Loading Dye (6×), DreamTaq Green DNA Polymerase, GeneRuler 1 kb DNA Ladder, methanol (HPLC purity), SYBR Safe DNA Gel Stain

Unilever, UK/Netherlands

Savo (sodium hypochlorite, commercial solution)

3.1.3 Laboratory equipment

Analytical balances ABT120-5DNM	Kern, Germany
Analytical balances BP160P	Sartorius, Germany
Autoclave Vaposteri	BMT Medical Technology, Czech Republic
Biological thermostat BT 120	Laboratorní přístroje Praha, Czech Republic
Centrifuge 5430 R, rotors A-2-MTP, and FA-45-24-11-HS	Eppendorf, Germany
Centrifuge MiniSpin plus, rotor F-45-12-11	Eppendorf, Germany
Cooling trap LT 105	Christ, Germany
Disposable Polypropylene, RNase-Free Pellet Pestles	DWK Life Sciences, Germany
Disposable 1.5 mL polystyrene spectrophotometer cuvettes	Kartell Labware, Italy
Holten Horizontal Laminar Airflow Clean Bench 1.5 Plus	Thermo Fisher Scientific, USA
HPLC column Luna C18(2) 3 µm, 150×2 mm	Phenomenex, USA
HPLC system UltiMate 3000	Dionex, USA
-20 °C Labtop cooler Nalgene	Sigma-Aldrich, USA
LightCycler 480 System	Roche, Switzerland
Manual single channel pipettes	Eppendorf, Germany
Mastercycler ep gradient S	Eppendorf, Germany
Minishaker MS2	IKA-Werke, Germany
Mixing Block MB-102	Bioer, China
Mass spectrometry detector 3200 QTRAP LC/MS/MS	Applied Biosystems, USA
NanoDrop One^{c}	Thermo Fisher Scientific, USA

Oasis MCX 1cc (30 mg) extraction cartridges (1 mL)	Waters, USA
Owl EasyCast B1 Mini Gel Electrophoresis	Thermo Fisher Scientific, USA
pH meter Orion Star A211	Thermo Fisher Scientific, USA
Plant Growth Chamber MLR 350 H	Sanyo, Japan
Precision balance EK-200	A&D, Japan
Rotary vane pump RV3	Edwards, UK
Scanner Perfection V700 Photo	Epson, Japan
spectrophotometer Helios Alpha	Thermo Electron, USA
Standard Power Pack P25	Biometra, Germany
Transilluminator TI1	Biometra, Germany
Ultra-low temperature freezer MDF-U74V	Sanyo, Japan
Universal hot plate magnetic stirrer RCT basic	IKA-Werke, Germany
Vacuum concentrator Alpha RVC CMC-2	Christ, Germany
Visiprep SPE Vacuum Manifold	Supelco, USA
Water heater thermostat W21	MLW, Germany

3.2 Methods

3.2.1 Oat leaf senescence assay

One of the well-known physiological effects of CKs is delayed senescence. Detached leaves belong to elementary experimental models used for decades. Different assays were developed with regards to model plants and the tested hypotheses [24, 135-140]. For our purposes, the methodology was adopted and modified according to [141]: the senescence in cultivated first fully developed oat leaves was measured spectrophotometrically based on a decrease in chlorophyll content.

Oat (*Avena sativa* L. cv. Patrik) seeds were soaked for 12 hours in aerated distilled water and sown into perlite saturated with 2-fold concentrated Knop's nutrition solution (1 L contains 2 g calcium nitrate, 0.5 g monopotassium phosphate, 0.5 g magnesium sulphate, 0.24 g potassium chloride, 2 drops of 5% ferric chloride solution, w/v; pH was adjusted to 5.7 with 1 M potassium hydroxide or 1 M hydrochloric acid). Plants were cultivated for 10 days in a growth chamber under controlled long-day conditions (18 h light at 20 °C/6 h dark at 18 °C) and 90% humidity. The first fully developed leaves were cut to 7 cm long apical segments. Analyses performed on four sample segments without any additional treatment are labeled as fresh blank. For further treatment, basal ends of four segments were placed into 15 mL polystyrene tubes with 1 mL of test solution or water (in case of cultivated blank). Tested compounds (*tZ*, *tZ*7G, *tZ*9G, iP, iP7G, iP9G) were dissolved into 0.1 M hydrochloric acid, diluted and neutralized with 0.1 M sodium hydroxide (final sodium chloride concentration varied from 0.04 to 0.24 M) and applied in 7 concentrations: $3.2 \cdot 10^{-8}$ M, $1.6 \cdot 10^{-7}$ M, $8 \cdot 10^{-7}$ M, $4 \cdot 10^{-6}$ M, $2 \cdot 10^{-5}$ M, $1 \cdot 10^{-4}$ M, and $5 \cdot 10^{-4}$ M. The test tubes in six replicates were incubated at 27 °C in darkness for four days. Three replications were used for chlorophyll extraction and the other three were used for determination of CK profiles (see chapter 3.2.2, p. 38).

Chlorophyll was extracted from oat leaf segments according to [141] with slight modifications by 10 mL 80% ethanol (v/v) in an 80 °C water bath (the heating time 15 min). The optical absorbance of leaf extracts was measured at wavelength 665 nm on spectrophotometer Helios Alpha. Chlorophyll retention was displayed as absorbance/fresh weight (FW) ratio percentage of fresh levels in non-cultivated oat leaf segments (fresh blank, value 100 %). Results shown consist of as average values from at least three biological replicates accompanied by standard deviation.

3.2.2 Cytokinin profiles analysis

CKs content was determined according to the method developed in the Institute of Experimental Botany CAS [142]. This method consists of following basic single steps: homogenization of plant material, extraction, purification, and quantification of phytohormones. A crucial part of the first step is a quantitative release of phytohormones from plant tissue into extraction solvent. Concurrently separation of enzymes catalysing their metabolic conversions and even degradation should be accomplished. Altogether, the extraction solvent precipitating proteins and low temperature during the process is required. For later quantification, samples are enriched with internal standards (compounds with a similar chemical structure to the analytes and with known purity and concentration). The second step – purification is achieved by solid phase extraction (SPE) on columns containing mixed-mode cation exchanger (MCX). It enables both reversed phase and cation exchange extraction and, with properly selected solvents, separates phytohormones in two fractions (hydrophobically bond – i.e. auxin vs. electrostatically bond – CKs). Last step (quantification) is achieved by high-performance liquid chromatography (HPLC) combined with mass spectrometry [143].

Dried oat leaf segments (both fresh and cultivated) were homogenized with mortar and pestle in liquid nitrogen. For reliable analysis and statistically robust results, we used at least three biological replicates. Endogenous CKs were extracted from 50 – 100 mg plant material (FW) by addition of 500 μL cold modified Bieleski's solution (methanol/formic acid/water in ratio 15/1/4, v/v/v). Each sample was then enriched with 50 μL internal CKs standards – methanol/water (1/1, v/v) solution of ^{2}H-labelled CKs in concentration 0.2 μL each (100 pmol of $^{2}H_5$-*tZ*, $^{2}H_5$-*tZ*R, $^{2}H_5$-*tZ*7G, $^{2}H_5$-*tZ*9G, $^{2}H_5$-*tZ*OG,$^{2}H_5$-*tZ*ROG, $^{2}H_5$-*tZ*RMP, $^{2}H_3$-DHZ, $^{2}H_3$-DHZR, $^{2}H_3$-DHZ9G, $^{2}H_6$-iP, $^{2}H_6$-iPR,

$^{2}H_{6}$-iP7G, $^{2}H_{6}$-iP9G, $^{2}H_{6}$-iPRMP were added to the samples). Mixed samples were left for overnight extraction in -20 ℃. Solids were removed by centrifugation (Eppendorf 5430 R, 30 000×g, 20 min, 4 ℃) followed by supernatant collection into new sample tubes. The pellets were reextracted with additional 500 μL of cold modified Bieleski's solution in -20 ℃ for 30 min. The supernatant after re-centrifugation (same conditions as before) was fused with previous stock and stored at -80 ℃ for 20 min. The volume of pooled supernatants was reduced to approximately 200 μL with rotary vacuum evaporator: 40 ℃, ideal pressure 15 Pa, safety pressure 115 Pa (Alpha RVC CMC-2, rotary vane pump RV3, cooling trap LT 105). The samples were diluted with 500 μL 1 M formic acid and loaded to equilibrated SPE column (Oasis MCX mounted on vacuum manifold; 1 mL methanol flush followed by 1 mL formic acid flush). Two additional washes with 500 μL 1 M formic acid were used to transfer all CKs from the sample tubes to SPE columns. The columns were then washed with 500 μL 1 M formic acid and 250 μL of distilled water. Acid phytohormones (e.g. auxins, abscisic acid, jasmonates, and gibberellins) were eluted with two rinses of 250 μL methanol (if isotope-labelled internal standards for these hormones were applied, their quantification might be performed; we focused strictly to CK fraction thus flow-through was discarded). The second fraction eluting CKs was performed with two rinses of 250 μL 0.35 M ammonium hydroxide in 60% methanol and collected. After short freezing (-80 ℃ for 20 min), eluates were evaporated at a rotary vacuum evaporator (conditions described above). Dried samples were dissolved into 30 μL 5 mM acetic acid in 95% acetonitrile in water, frozen (-80 ℃ for 20 min) and centrifugated (Eppendorf 5430 R, 30 000×g, 20 min, 4 ℃). Supernatants were transferred into autosampler vials and analysed on HPLC/mass spectrometry system (UltiMate 3000, column Luna C18(2) 3 μm, 150×2 mm, 3200 QTRAP LC/MS/MS). Sample injection volume was 3 μL, elution flow

rate 0.3 mL min^{-1}, linear gradient of solvents A (5 mM acetic acid in 5% acetonitrile in water) and B (5 mM acetic acid in 95% acetonitrile): 5-40 % B for 15 min, 100 % B for 5 min, 10% B for 10 min (equilibration). Mass spectrometry was set in positive electrospray mode with ion source parameters +4 000 V, nebulizer gas 50 psi, heater gas 50 psi, curtain gas 20 psi, gas heater 500 °C. Specific parameters like declustering potential, *m/z* of precursor ion, *m/z* of product ion, and collision energy were adjusted for each compound and internal standard to mass spectrometry analyzer in multiple reaction monitoring mode. For the determination of calibration curve parameters, the appropriate number of calibration standards were injected. Samples were quantified in two technical replicates based on isotope dilution with calibration curve in software Analyst 1.5.1 (Sciex, USA). Results shown consist of average values obtained for at least three biological replicates, each processed in two technical replicates. Statistic evaluation includes standard deviation and power regression analysis processed in Microsoft Excel for CK dilution series.

3.2.3 Arabidopsis growth on cytokinin enriched media

Soon after their discovery, CKs were hypothesized to inhibit the root growth. This presumption was originally based on cultured tobacco callus root/shoot formation promoted by different auxin/CK ratio in the culture medium [23]. Further examination on CK affected inhibition of root growth proved concentration dependence and led to the development of numerous bioassays comparing effectiveness of different CK substances [144-149].

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia) plants were grown on half-strength MS media (1 L contains 10 g agar, 10 g sucrose, and 2.3 g MS salts in distilled water; pH was adjusted to 5.7 with 1 M potassium hydroxide or 1 M hydrochloric acid) enriched with selected CKs. Because of a thermal instability of used CKs (*tZ*, *tZ*7G, *tZ*9G, iP, iP7G,

and iP9G), the medium was firstly autoclaved (Vaposteri, BMT Medical Technology), then cooled to approximately 50 °C and after that a respective volume of CK solution (concentration 1 mM in DMSO) was added. The mixed media were poured to square Petri dishes (11.8×11.8 cm; approximately 50 mL per dish) and left for solidification.

Arabidopsis seeds were sterilized by 1 mL 70% ethanol (v/v) for 2 min, then by 1 mL 10% Savo (v/v; sodium hypochlorite commercial solution) with 1 drop of Tween 20 for 10 min and rinsed twice with 1 mL sterile water. Seeds were then sown on prepared media; sealed Petri dishes were stratified in 6 °C and darkness for 3 days and then moved to growth chamber (16 h light/8 h dark, 20 °C). Scans were taken on the eighth (11 days after sowing i.e. 11 DAS) and the eleventh day of light cultivation (14 days after sowing, i.e. 14 DAS), respectively. Seedlings were harvested at the latest time point as described below. Primary root length was measured on scans with software Fiji (distribution of ImageJ) [150]. Statistic evaluation was performed in RStudio on more than 20 samples for each treatment. Outliers were identified by Grubbs test and respective seedlings were not used for transcription analysis. Similarly, also Shapiro-Wilk test, unpaired two-tailed Student's t-test and Tukey multiple comparison test were used.

3.2.4 Sampling, RNA isolation

Observed physiological differences in plants exposed to exogenous CKs in cultivation media are partly a manifestation of changed gene expression. For further investigations, RNA from Arabidopsis seedlings grown on media specified above (see chapter 3.2.3, p. 40) was isolated with commercial RNeasy Plant Mini Kit. This well-established procedure combines selective binding properties of a silica-based membrane with the speed of microspin technology. Plant material disruption and lysis is ensured by guanidine-thiocyanate–containing buffer. Ethanol addition establishes ideal RNA binding conditions to an RNeasy Mini spin column

and allows contaminants removal. Finally, RNA shorter than 200 nucleotides (membrane size exclusion limit) is eluted in water [151].

The 14 days-old Arabidopsis plants grown on media described above were cut between hypocotyl and epicotyl to obtain root and shoot segments. Plant segments were harvested separately, blotted dry, weighed and frozen in liquid nitrogen. Plant material was quickly homogenized with RNase-Free Pellet Pestles directly in the test tube in the labtop cooler. The RNA isolation was performed with the RNeasy Plant Mini Kit using sterile, RNase-free material (such as solutions and pipettes tips). Producer's protocol [151] was followed: 450 μL lytic Buffer RLT with 2-mercaptoethanol was added to tissue powder, the sample was vortexed and incubated in 56 °C for 3 min (Mixing Block MB-102). The lysate was then transferred to a QIAshredder spin column placed in 2 mL collection tube and centrifuged (MiniSpin plus, 12 000×g, 2 min). The supernatant of the flow-through was transferred to a new tube with half of its volume (usually 225 μL) 96% ethanol (v/v) and mixed by pipetting. Prepared sample was immediately transferred to an RNeasy spin column placed in a 2 mL collection tube and centrifuged (MiniSpin plus, 6 500×g, 15 s). After this and the following three centrifugations, the collection tube was emptied and reused. RWI buffer (700 μL) was added to the column and centrifuged (MiniSpin plus, 6 500×g, 15 s). The column membrane was twice rinsed with 500 μL RPE buffer followed by centrifugation (MiniSpin plus, 6 500×g, firstly 15 s, secondly 2 min). After that, the RNeasy spin column was placed in a new 2 mL collection tube and the column membrane was dried with centrifugation (MiniSpin plus, 12 000×g, 1 min). The RNeasy spin column was placed in a new 1.5 mL collection tube and the bound RNA was eluted with 30 μL RNA-free water by centrifugation (MiniSpin plus, 6 500×g, 1 min). The RNA solutions were stored at -20 °C and treated on ice plate (to prevent possible degradation).

3.2.5 RNA purification, quantification and visualization

To prevent simultaneous detection of both DNA and RNA levels in samples, DNA degradation step is an essential component of RNA processing. For this treatment, we used the DNA-free Kit. This commercial solution is designed to digest contaminating DNA by the recombinant DNase I to levels below the limit of detection by routine PCR [152]. The effect of this treatment might be observed by spectrophotometry: measurement of absorbance at 260 nm is a well-established method for estimation of nucleic acid concentration [153]. To exclude the effect of contaminants (in this case DNA, proteins, phenol and organic salts from isolation kit), NanoDrop One^{c} in RNA quantification mode performs measurements at wavelengths range 190 – 850 nm. Built-in Acclaro Sample Intelligence Technology determines automatically sample RNA concentration based on measured absorbance [154]. The samples with low RNA content, as well as those strongly contaminated, were excluded from further investigations. The nucleic acid integrity was visualized by electrophoresis on 1.8% (w/v) agarose gel.

For the spectrophotometric quantification and RNA visualization by electrophoresis, 4 µL of isolated RNA solutions were taken. The remaining 26 µL were treated with the DNA-free Kit: 2.6 µL 10×DNase I buffer and 1 µL rDNase I were added to the sample and gently mixed. After 45 min of incubation in 37 °C heating block, 2.6 µL resuspended DNase Inactivation Reagent was added. The samples were then kept for 2 min at room temperature and mixed occasionally. Samples were centrifugated (MiniSpin plus, 8 117×g, 2 min) and the supernatants (final RNA solutions) transferred into fresh tubes without disruption of the pellets.

For a visual comparison of final and preliminary RNA solutions, electrophoresis on 1.8% (w/v) agarose gel was performed. The gel (for 12 samples before/after DNase treatment)

was prepared by heating 1.26 g agarose in 70 mL 1×TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA water solution, pH adjusted to 8, prepared from Sigma concentrate), nucleic acid intercalating staining (7 μL 10 000×SYBR Safe DNA Gel Stain) was mixed in after cooling to approximately 60 °C immediately before pouring gel into electrophoretic tray. Prepared samples (3 μL RNA solution, 1 μL 6×DNA loading dye, 5 μL deionized water) were loaded to the solidified gel together with GeneRuler 1 kb DNA Ladder (1 μL DNA ladder, 1 μL 6×DNA Loading Dye, 4 μL deionized water). Electrophoresis, performed in Owl EasyCast B1 Mini Gel Electrophoresis chamber, ran in 1×TAE buffer under initial (first 10 min) voltage 4 V cm^{-1} increased to later 6 V cm^{-1}. Nucleic acids were visualized with transilluminator TI1 and recorded with a common camera.

RNA contents in 1 μL both final and DNase not-treated RNA solutions were measured by spectrophotometer NanoDrop One[c] in RNA quantification mode.

3.2.6 Reverse transcription polymerase chain reaction (RT-PCR)

To quantify gene expression the amplification of individual RNA molecules by combining reverse transcription and the polymerase chain reaction (RT-PCR) might be used [155]. A reverse transcriptase, a key enzyme in RT-PCR, synthesizes DNA strand complementary to RNA. By using poly-T oligonucleotide as a primer, we obtained cDNA (complementary DNA) from all mRNAs present in samples [156].

RNA extracted and purified in previous steps was reversely transcribed by Promega Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV Reverse Transcriptase). The assay was performed with 13 μL RNase free water solution of 2 μg RNA (the volume of final RNA solution for dilution to intended concentration was counted from sample measurement

at Nanodrop One[c], see 3.2.5, p. 43). Producer's protocol [157] was slightly modified with regards to chemicals available (reagents are listed in Table 2).

The procedure included 5 min preincubation of RNA template and primers at 75 °C, immediate cooling on ice, RT-PCR premix addition and Mastercycler ep gradient S incubation (42 °C/60 min, 70 °C/5 min, 10 °C cooling till further manipulation).

Table 2: Reagents used for reverse transcription

Reagent		Volume per reaction
2 µg RNA template in RNase free water		13 µL
oligo $d(T)_{18}$ (5 µM)		1 µL
RT-PCR premix	M-MLV Reverse Transcriptase	1 µL
	M-MLV 5× Reaction Buffer	5 µL
	RNA inhibitor RNAsin	0.5 µL
	dNTP solution (10 mM each)	1.25 µL
	RNase free water	3 µL

Obtained cDNA was divided in stock solution (19.75 µL stored at -80 °C) and qPCR samples (5 µL cDNA were 20× diluted – to final volume 100 µL).

3.2.7 Real-time quantitative polymerase chain reaction (qPCR)

The quantitative polymerase chain reaction (qPCR) relies on a direct relationship between the rate of product generation and original DNA concentration (or similarly cDNA and mRNA concentration). Using this method, expression levels of several genes related to CKs metabolism were compared to those of housekeeping genes. Designed primers were tested by conventional PCR followed with electrophoresis on 1.2% (w/v) agarose gel. The detection of only one PCR product was verified by heat dissociation after the final PCR cycle (single peak in a melting temperature was desired) [155].

Genes and respective primers used for qPCR analysis are listed in detail in Table 3. Primer sequences published in literature were used for genes *ABCG14* [131], *ACT2* [158], *ARR5* [34], *ARR7* [118], *EF1ALPHA* [34], and *IPT5* [158]. Primers for genes *AHK4*, *ARR16*, and *CRF5* were designed with PrimerQuest tool [159] and synthesized by Sigma-Aldrich. *IPT9*, *UGT76C1*, *UGT76C2*, and *UGT85A1* primers were kindly provided by Mgr. Mária Škrabišová, Ph.D. (Faculty of Science, Palacký University in Olomouc).

Table 3: qPCR primer characterization

Gene locus	Primer sequence (5'->3') F - forward, R - reverse	Length [bp]	Melting temperature [°C]	Product length [bp]
ABCG14	F: TCGGTGCTCTGCTTATGAAC	20	57.99	112
NM_102911	R: ACGATGAAGGGAGGAATTTG	20	55.42	
ACT2	F: CTTGCACCAAGCAGCATGAA	20	59.68	68
NM_112764	R: CCGATCCAGACACTGTACTTCCTT	24	61.89	
AHK4	F: AGTGGGATGGATGGTTAC	18	53.06	99
NM_179594	R: CGACGAAGGTGAGATAGG	18	53.96	
ARR5	F: CTACTCGCAGCTAAAACGC	19	56.85	108
NM_114679	R: GCCGAAAGAATCAGGACA	18	54.61	
ARR7	F: CTGGCATTGAGTAATCCGTCACTATC	26	61.5	120
NM_101763	R: TGACGACTGTAGAGAGTGGAACTAGG	26	62.96	
ARR16	F: CGTTATGAAGGTGAGTCTAATC	22	54.17	85
NM_001202793	R: CTCCTGCTTCACTTTCTTG	19	53.62	
CRF5	F: CATGCGCTACTGATTCTTC	19	54.02	87
NM_130194	R: ACCTGATCTCATCCACATAA	20	53.09	
EF1ALPHA	F: TGAGCACGCTCTTCTTGCTTTCA	23	63.15	76
NM_001125992	R: GGTGGTGGCATCCATCTTGTTACA	24	63.16	
IPT5	F: ACTTGTTTGCTTGCGTGTA	19	55.99	325
NM_001343584	R: CCTTCTTTCGTCATCGTC	18	52.92	
IPT9	F: GGATTGTATCTGCGATGGTTTATGT	25	59.53	79
NM_001203415	R: CATGGGCCTCAGCGATAACT	20	59.89	
UGT76C1	F: CATATCGATCTTTAGATGAGTT	22	51.05	119
NM_120669	R: GTTCTATAATCAATTGACAAGAG	23	51.16	
UGT76C2	F: CGAGAAATCGGTCAAACAAG	20	55.05	110
NM_120668	R: AAACGTTGAGGTATCTTTAGC	21	53.96	
UGT85A1	F: GACTTCCTTCGTTCCGATTT	20	55.79	218
NM_102089	R: CTCCGCAACATCAAGAGTAA	20	55.50	

Suitability of designed primers was tested by electrophoretic analysis of PCR products. Selected qPCR samples (2 μL each) were mixed with 13.7 μL deionized water, 2 μL 10× Dream Taq Buffer, 1 μL primer solution (forward and reverse, both in 10 mM concentration), 0.2 μL dNTP solution (10 mM each), and 0.1 μL DreamTaq DNA Polymerase. PCR was performed in Mastercycler ep gradient S with the program specified in Table 4.

Table 4: PCR settings for primer testing

Programme	Number of cycles	Target temperature [°C]	Duration [s]
Pre-incubation	1	95	120
Amplification	35	95	45
		58	30
		72	15
Final elongation	1	72	300
Final hold	1	4	600

Agarose gel for electrophoresis (1.2% w/v) was prepared as specified above (chapter 3.2.5, p. 43) with agarose weighting modified accordingly. Obtained test DNA samples were applied to the gel as described in chapter 3.2.5, p. 43 electrophoretic conditions and further procedure remained the same.

Quantitative PCR was performed with GoTaq qPCR Master Mix according to the producer's instructions for use [160]. Each well of 96-well plate contained 2.5 μL qPCR sample, 1.5 μL deionized water, 0.5 μL forward primer solution and the same amount of reverse primer solution, and 5 μL 2× GoTaq qPCR Master Mix. The reaction was performed in the LightCycler 480 System, the temperature profile is shown in Table 5, p. 48. Reaction

rate was monitored by SYBR Green I fluorescence measured at the end of each elongation phase. Upon excitation by 465 nm light, the intensity of emitted 510 nm light was measured.

Table 5: qPCR settings

Programme		Number of cycles	Target temperature [°C]	Duration [s]	Ramp Rate [°C s^{-1}]
Pre-incubation		1	95	120	4.4
Amplification	denaturation	45	95	10	4.4
	annealing		58	15	2.2
	elongation		72	20	4.4
Melting curve		1	95	5	4.4
			65	60	2.2
Cooling		1	40	30	2.2

Results were processed using the software LightCycler 480 (Roche) and obtained C_p values (Crossing point) were used to determine relative expression. C_p is defined as the cycle number at which the fluorescence signal crosses a threshold. The fold-change of relative expression was calculated as:

$$\text{fold-change} = 2^{-\Delta\Delta C_P} = 2^{-(\Delta C_{P\,\text{treatment}} - \Delta C_{P\,\text{control}})}$$

While the ΔC_P is defined as a difference of $C_{P\ \text{target gene}}$ and a geometric average of $C_{P\ \text{ACT2}}$ and $C_{P\ \text{EF1ALPHA}}$. Data represent at least two technical replicates of biological samples consisting of approximately 20 seedlings sections (roots or shoots). For manual calculation in Microsoft Excel, technical replicates of treatment and control were paired one to one and results are shown as average fold-change values. Standard deviation was calculated from individual $\Delta C_{P\ \text{treatment}}$ values.

4 Results

4.1 Effect of exogenously applied cytokinins on senescing oat leaf segments

Our previous study suggested differential effect of *N7*- and *N9*- glucosides of *tZ* on dark-induced chlorophyll retention in oat leaf segments. The CK profiling revealed elevated concentrations of endogenous metabolites *cZ*OG and, in lesser extent, also *tZ*OG following *tZ*9G but not *tZ*7G application. Hypothetical metabolic conversion of a putatively "inactive" *tZ*9G to *O*-glucosides (and/or other products) was tested now in a different oat cultivar (*Avena sativa* L. cv. Patrik compared to previously used cv. Abel). In addition, *N7*- and *N9*-glucosylated derivatives of iP as a CK substance lacking hydroxylated side-chain necessary for a potential transglucosylation were included in the tests. To conclude, the effects of exogenously applied CK free bases *tZ* and iP and their corresponding *N*-glucosides (*tZ*7G, *tZ*9G, iP7G, and iP9G) on senescence of oat leaf segments cv. Patrik were examined.

4.1.1 Oat leaf senescence bioassay

Data demonstrating chlorophyll retention in oat leaf segments after exogenous CK application of tested CK derivatives are summarized in Figure 5, p. 50. While none of the tested iP-type CKs (including iP itself) affected chlorophyll retention, the *tZ* free base and its *N9*-glucoside, *tZ*9G, were shown to delay senescence in higher concentrations ($2 \cdot 10^{-5}$ M and $1 \cdot 10^{-4}$ M). The highest effect on senescence retardation was found for $1 \cdot 10^{-4}$ M concentrated solutions of *tZ*9G preserving 77 % of initial chlorophyll content compared to the control (cultivated blank whose chlorophyll content decreased to 38 % of initial level) and *tZ* (which preserved 74 % of initial chlorophyll content). It should be kept in mind that the highest applied concentration ($5 \cdot 10^{-4}$ M) of *tZ* and *tZ*9G induced necrosis on basal parts of leaf segments (manifested by changed colour and leaf structure).

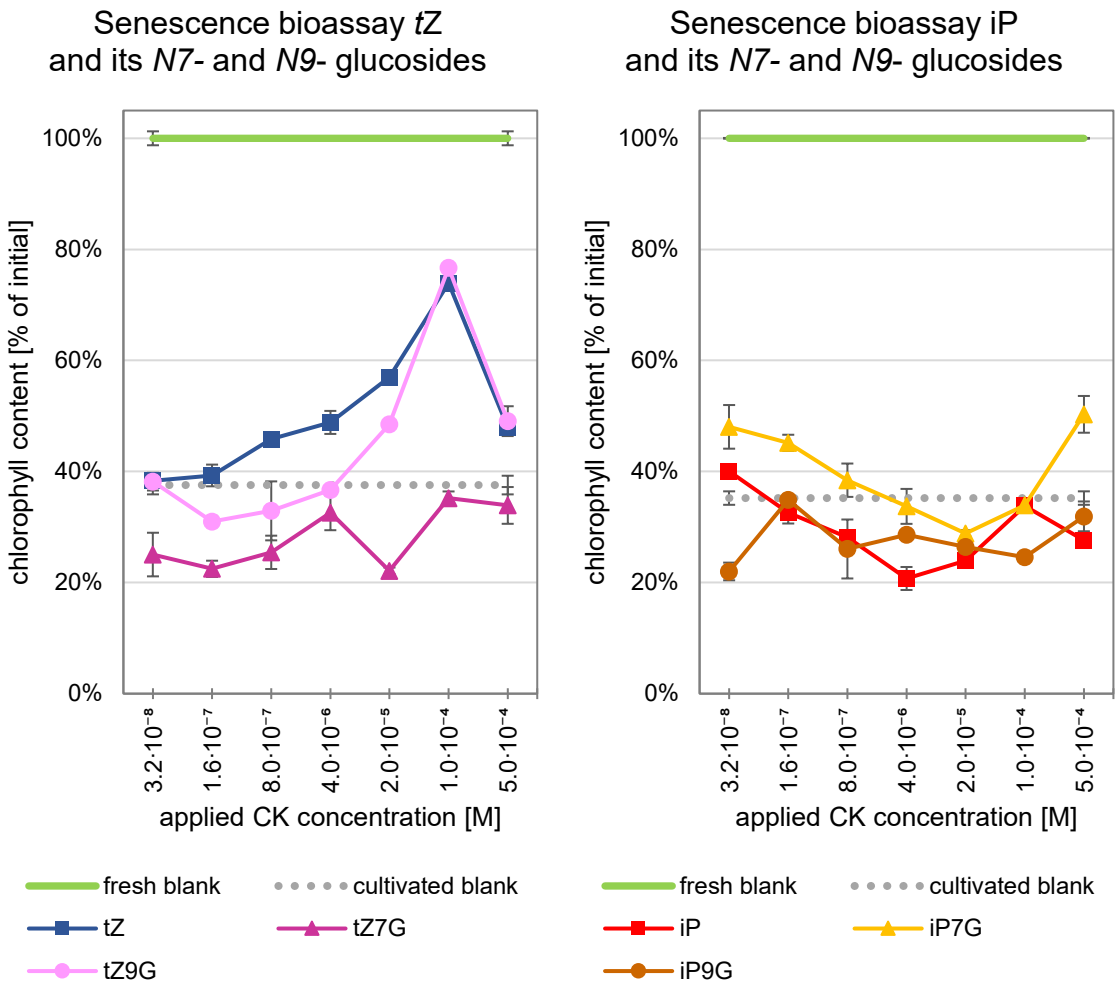


Figure 5: Comparison of effects of iP-and tZ-type cytokinins on dark-induced chlorophyll retention in senescing oat (Avena sativa L. cv. Patrik) leaf segments.

Values are expressed as percentage of the initial chlorophyll content (100 % is amount of chlorophyll in fresh leaves). Data represent average values of at least three replicates, standard deviation is demonstrated for each value.

4.1.2 Cytokinin profiles

Taking advantage of HPLC-mass spectrometry, the following profiles of endogenous CKs were revealed in control (both fresh and cultivated blank) and CK-treated (*tZ*, *tZ*7G, *tZ*9G, iP, iP7G, and iP9G) oat leaf segments (see Table 6, p. 51). From the data collected, interesting trends were noticed in the total CK content in oat leaf segments after the treatment (see Table 7, p. 52, and Table 9, p. 53).

Table 6: Endogenous cytokinin (CK) profiles in oat (Avena sativa L. cv. Patrik) leaf segments after exogenous CK application (tZ, tZ7G, tZ9G, iP, iP7G, and iP9G were applied in concentration $5 \cdot 10^{-4}$ M). Endogenous CK levels are expressed in pmol/gFW, standard deviation is shown in brackets for each value.

CK levels (SD)	Applied CK						Fresh blank	Cultivated blank
	tZ	tZ7G	tZ9G	iP	iP7G	iP9G		
tZ	22 463.8 (14 721.2)	139.3 (54.4)	330.2 (22.2)	357.3 (38.8)	49.8 (28.6)	70.1 (5.0)	2.2 (0.5)	3.6 (1.1)
tZR	277.1 (372.1)	0.1 (0.1)	0.3 (0.1)	5.3 (4.7)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
tZ7G	31.0 (17.6)	109 534.8 (10 046.2)	78.6 (7.7)	0.5 (0.9)	218.3 (32.6)	53.8 (1.7)	14.2 (9.7)	3.2 (0.7)
tZ9G	10 930.2 (1 509.2)	23.8 (18.2)	108 514.0 (3 028.2)	13.1 (8.0)	226.4 (44.8)	152.7 (10.7)	0.8 (0.5)	1.0 (0.4)
tZOG	14 694.6 (3 686.7)	161.3 (118.1)	24.3 (11.7)	88.1 (16.3)	4.1 (3.4)	2.7 (2.9)	20.0 (8.3)	7.0 (5.2)
tZROG	553.6 (341.5)	0.8 (1.1)	1.8 (0.7)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
tZRMP	607.8 (841.1)	1.8 (1.6)	1.7 (1.5)	18.7 (4.2)	2.0 (0.4)	1.9 (2.4)	0.0 (0.0)	0.4 (0.7)
DZ	470.7 (581.3)	2.8 (0.6)	0.0 (0.0)	23.8 (4.4)	2.4 (1.4)	0.7 (0.3)	0.4 (0.0)	0.7 (0.1)
DZR	56.1 (81.1)	0.1 (0.3)	0.0 (0.0)	1.2 (0.6)	0.0 (0.0)	0.1 (0.2)	0.2 (0.2)	0.0 (0.0)
DZ7G	0.5 (0.3)	114 (48.5)	0.0 (0.0)	0.5 (0.4)	1.0 (0.9)	0.4 (0.4)	4.1 (2.3)	1.6 (1.1)
DZ9G	6 097.0 (1 063.4)	3.5 (4.8)	440.4 (57.5)	107.7 (54.5)	50.4 (26.9)	75.0 (19.0)	2.8 (1.3)	2.9 (2.8)
DZROG	53.0 (47.8)	2.9 (3.0)	8.1 (6.1)	0.9 (1.6)	9.3 (4.7)	9.3 (2.9)	0.0 (0.0)	5.9 (5.2)
DZRMP	78.8 (124.2)	0.9 (0.8)	0.0 (0.0)	3.0 (2.6)	0.0 (0.0)	1.0 (1.7)	0.8 (0.8)	0.0 (0.0)
cZ	45.4 (64.7)	3.6 (2.5)	1.1 (1.0)	279.4 (27.6)	16.4 (5.1)	4.2 (1.9)	0.4 (0.0)	3.8 (3.7)
cZR	3.8 (5.5)	2.6 (1.6)	0.9 (1.1)	5.9 (2.4)	25.9 (15.5)	14.7 (12.4)	0.3 (0.2)	1.5 (1.1)
cZ7G	2.7 (2.5)	637.4 (526.5)	48.2 (1.4)	1.0 (0.9)	73.1 (5.5)	0.3 (0.3)	0.6 (0.2)	0.3 (0.1)
cZOG	182.2 (98.7)	52.6 (17.2)	57.2 (29.7)	201.8 (43.4)	109.2 (9.8)	119.7 (15.4)	103.8 (17.4)	111.0 (4.9)
cZ9G	37.3 (30.2)	2.7 (1.6)	0.0 (0.0)	112.4 (53.4)	592.7 (260.3)	794.4 (42.9)	0.5 (0.1)	0.6 (0.6)
cZROG	198.2 (31.2)	103.4 (18.5)	70.2 (9.6)	333.8 (51.6)	143.1 (39.3)	147.5 (36.9)	141.1 (48.4)	165.3 (34.9)
cZRMP	10.9 (18.9)	1.5 (1.3)	0.0 (0.0)	15.9 (3.3)	2.0 (0.4)	1.9 (2.4)	0.0 (0.0)	0.8 (1.4)
iP	26.2 (20.2)	24.1 (29.0)	1.1 (0.7)	28 127.8 (6 986.6)	901.8 (323.6)	231.2 (66.3)	31.1 (11.3)	200.2 (13.2)
iPR	0.3 (0.3)	0.2 (0.3)	0.1 (0.2)	185.5 (191.0)	14.8 (23.7)	1.2 (2.1)	0.3 (0.5)	0.0 (0.0)
iP7G	8.2 (1.1)	54.6 (52.5)	25.8 (3.6)	0.0 (0.0)	387 714.5 (32 978.3)	613.0 (521.8)	0.9 (0.4)	4.0 (4.8)
iP9G	6.2 (4.7)	3.7 (4.0)	1.0 (0.2)	11 144.4 (3 821.3)	68 133.2 (27 201.8)	103 004.5 (1 738.8)	0.1 (0.1)	5.8 (4.2)
iPRMP	0.8 (0.3)	0.7 (0.7)	0.5 (0.4)	1 053.2 (266.1)	6.2 (6.8)	2.2 (0.4)	0.6 (0.2)	0.7 (0.7)

The cultivation in darkness increases the endogenous CK levels in detached oat leaves (see Table 7 and Table 8). While observing a significant decrease in *tZ*-type CKs (40 % of fresh content), the level of DZ-type CKs remains approximately constant and total increase is caused by mounted iP-type CKs and partially also *cZ*-type CKs (see Table 7). The total increase in CK levels (195 pmol/gFW) responds almost exclusively to the elevated free bases form of CKs (174 pmol/gFW). Only minor changes in glucosides content were present (the amount of *N*-glucosides was slightly decreased, while *O*-glucosides were upregulated), ribosides and ribotides (see Table 8). This might be perceived as a compensation mechanism aimed to preserve the physiological state of leaves.

Table 7: Changes in endogenous cytokinin (CK) profile after incubation in oat (Avena sativa L. cv. Patrik) leaf segments based on the nature of the CK N^6-isoprenoid side-chain

CK content [pmol/gFW]	*tZ*-type	DZ-type	*cZ*-type	iP-type	total CK pool
Fresh blank	37.2	8.4	246.8	33.1	325.4
Cultivated blank	15.1	11.1	283.4	210.7	520.3
Difference caused by cultivation	-22.1	2.7	36.6	177.6	194.8

Table 8: Changes in endogenous cytokinin (CK) profile after incubation in oat (Avena sativa L. cv. Patrik) leaf segments according to their conjugation status and physiological function

CK content [pmol/gFW]	free bases	*N*-glucosides	*O*-glucosides	riboside (phosphate)s	total CK pool
Fresh blank	34.2	24.1	265.0	2.2	325.4
Cultivated blank	208.3	19.4	289.2	3.4	520.3
Difference caused by cultivation	174.1	-4.7	24.2	1.3	194.8

Table 9: The levels of endogenous cytokinins (CKs) and their proportional representation in the total cytokinin pool in oat (Avena sativa L. cv. Patrik) leaf segments after their exogenous application (CKs applied at 5 · 10^{-4} concentrations).

Applied CK	Its maximal concentration in segments [pmol/gFW]	Its percentage in total CK pool	Total CK pool in segments [pmol/gFW]
tZ	22 464	39.5	56 837
*tZ*7G	109 535	98.8	110 824
*tZ*9G	108 514	99.0	109 606
iP	28 128	66.8	42 081
iP7G	399 277	88.0	453 766
iP9G	103 005	97.8	105 302
Fresh blank			325
Cultivated blank			520

As shown in Table 6, p. 51 and Table 9, all exogenously applied CKs expectedly increased endogenous CK concentration in oat leaf segments. The tables show values for the highest concentration (5 · 10^{-4} M) for applied CK free bases *tZ* and iP and their corresponding *N*-glucosides (*tZ*7G, *tZ*9G, iP7G, and iP9G). According to the data, tested substances might be divided into 3 groups based on the induced effect: free bases (*tZ* and iP), highly susceptive glucoside (iP7G) and other glucosides (*tZ*7G, *tZ*9G, and iP9G) (see below).

In general, the leaf segments cultivation in darkness leads to increased total cytokinin levels. While cultivation in water causes approximately a 1.6-fold increase of the total CK content (520 *vs.* 325 pmol/g FW; Table 9), the increase of the total CK pool after application of 5 · 10^{-4} M iP7G is ca. 1 400-fold (453 766 *vs.* 325 pmol/g FW). In this regard, iP7G differs significantly from other studied glucosides with respect to the ability to penetrate leaf tissue. For further investigation, time-course measurements of CK levels were performed in addition to the original protocol – oat leaf segments (prepared as described in chapter 3.2.1, p. 37) incubation in either 5 · 10^{-4} M iP7G solution or water lasted 6, 12, 24,

and 96 hours (4 days), respectively. The time-course of CK contents revealed rapid iP7G uptake during the first 12 h of incubation which subsequently decelerated in time (Figure 6). The metabolic conversions had been detected particularly from 24 h onwards. Even though the levels of some CK derivatives were increased (especially *tZ*9G and *cZ*7G, see Table 12, p. 59), their low biological activity and late formation have not influenced the leaf senescence (which has been observed at the 6 h samples onward).

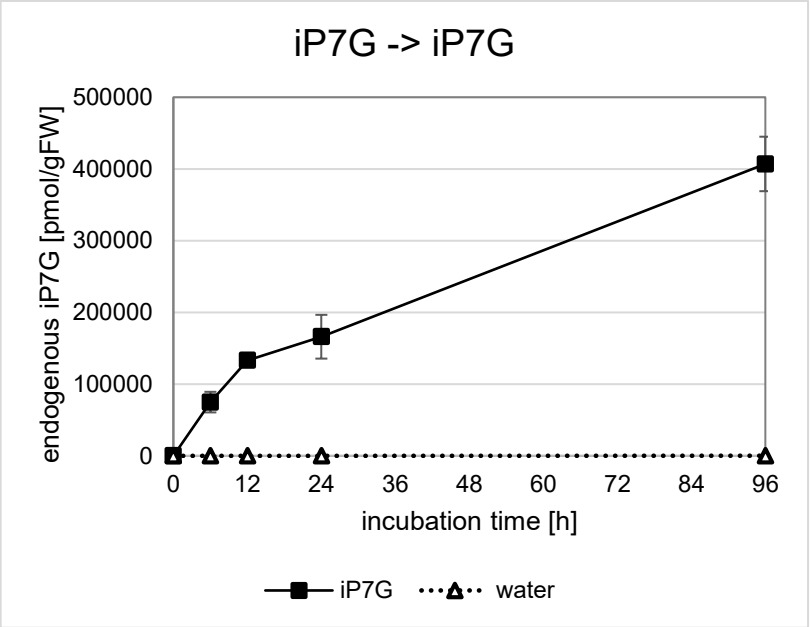


Figure 6: Dynamics of endogenous iP7G content in leaf segment cultivated in iP7G solution ($5 \cdot 10^{-4}$ M) and water

As expected, the endogenous amounts of applied substances were proportional to the test solution concentrations (e.g. Figure 7A, p. 55). The exceptions were observed after *tZ* and iP application. The *tZ* applied in lower concentrations ($3.2 \cdot 10^{-8}$ M, $1.6 \cdot 10^{-7}$ M, and $8 \cdot 10^{-7}$ M) was presumably faster degraded compared to higher concentrations ($4 \cdot 10^{-6}$ M, $2 \cdot 10^{-5}$ M, $1 \cdot 10^{-4}$ M, and $5 \cdot 10^{-4}$ M) (see Figure 7B, p. 55). The role of the enzymatic degradation catalysed by CKX in this effect is highly plausible. On the other hand, the response to iP application was not concentration-dependent (described below and shown in Figure 8A, p. 58).

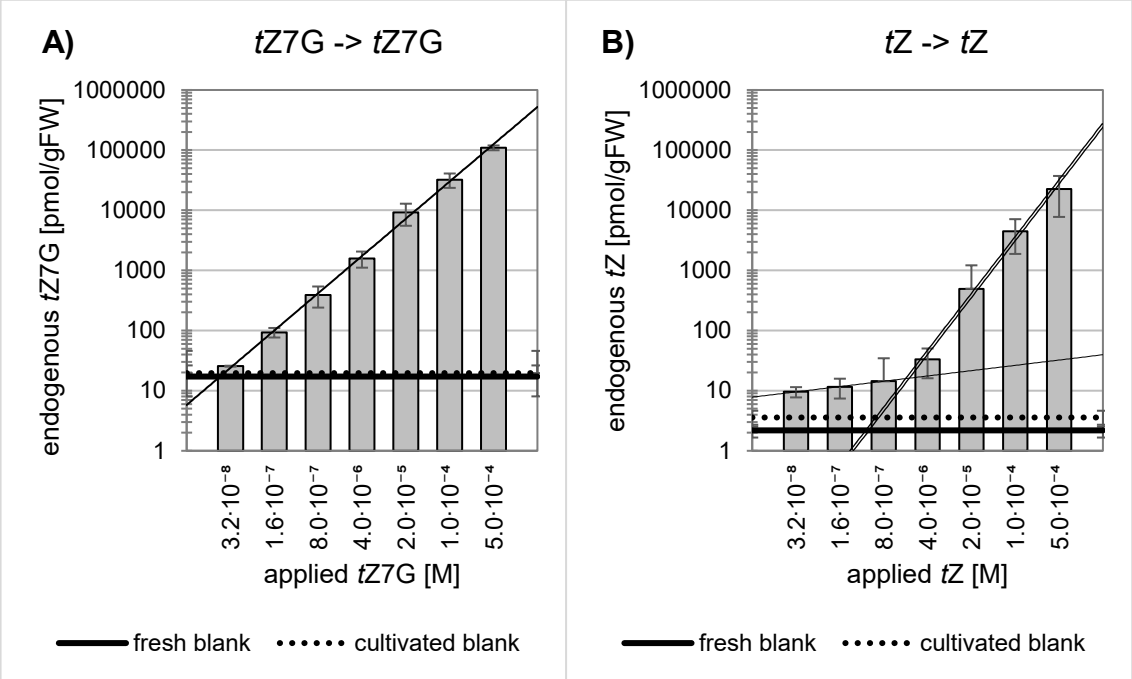


Figure 7: Increased amount of endogenous tZ7G (A) and tZ (B) in oat leaf segments after their exogenous application

While the endogenous levels of applied CKs were usually proportional to the test solutions concentration (as demonstrated by A: $y = 10^{8} \cdot c^{0.8862}$, $R^2 = 0.9982$), the endogenous tZ content (B) is not directly proportional to its applied concentration. The best fit was achieved when two equations were used: $y_1 = 84.24 \cdot c^{0.1262}$, $R_1^2 = 0.9989$ (for tZ concentrated $3.2 \cdot 10^{-8} – 8.0 \cdot 10^{-7}$), and $y_2 = 9 \cdot 10^{8} \cdot c^{1.3521}$, $R_2^2 = 0.9875$ (for tZ concentrated $4.0 \cdot 10^{-6} – 5.0 \cdot 10^{-4}$) respectively.

In the used model (*Avena sativa* cv. Patrik), similar changes in endogenous CK levels were observed after *tZ, tZ*7G, and *tZ*9G application as we reported previously for the different cultivar (*Avena sativa* cv. Abel). Exogenous *tZ* application causes an increase of endogenous levels of a wide range of CK metabolites, e.g. *tZ*R, *tZ*9G, *tZ*RMP, *tZ*ROG, *tZ*OG, DZ, and DZ9G (see Table 10, p. 56). These metabolic conversions do not include *cZ*- and iP-type CKs (see Table 11, p. 57).

Slight changes in endogenous CK profile were noticed upon cultivation in *tZ*7G solution (increased *cZ*7G and partially *tZ* and *tZ*OG). The *tZ*9G application affected a similar range of metabolites (increased levels of *tZ, tZ*7G, and DZ9G were found), but the elevation

of active form *tZ* was more pronounced. Contrary to our previous research on cultivar Abel, endogenous *cZOG* levels were not elevated, although delayed chlorophyll retention following *tZ*9G treatment was recorded. Our hypothesis regarding potential activity of *tZ*9G caused by its metabolic conversion to e.g. *cZOG* thus should be reconsidered with respect to potential physiological differences between the two cultivars, Abel and Patrik.

Table 10: Major changes in endogenous CK profiles after exogenous tZ, tZ7G and tZ9G application

** The coefficient of determination for all the applied concentrations without regard to differential metabolic turnover as shown in Figure 7B, p. 55.*

Applied $5 \cdot 10^{-4}$ M cytokinin (CK) solution	Triggered change of endogenous CK level	Detected metabolite content [pmol/gFW]	Fold-change compared to cultivated blank (if detected in cultivated blank)	Coefficient of determination for power regression
tZ	*tZ*	22 464	6 275	0.8941*
	tZR	277	———	0.8496
	*tZ*9G	10 930	11 268	0.9309
	tZRMP	608	1 483	0.9185
	tZROG	554	———	0.7451
	tZOG	14 695	2 108	0.9662
	DZ	471	636	0.8270
	DZ9G	6 097	2 102	0.9805
*tZ*7G	*tZ*7G	109 535	5 597	0.9982
	tZ	139	62	0.6697
	tZOG	161	36	0.9156
	*cZ*7G	637	2 055	0.8711
*tZ*9G	*tZ*9G	108 514	31 362	0.9995
	tZ	330	154	0.8964
	*cZ*7G	48	178	0.9052
	DZ9G	440	254	0.9680

As mentioned above, exogenous application of *tZ* induced especially changes in endogenous *tZ*-type CKs and to a lesser extent DZ-type CKs (see Table 11). Despite the extensive *tZ* metabolization (compared to its glucosides, see Table 10, p. 56), only the metabolic flow from tZ-type CKs to DZ-type CKs (presumably mediated by zeatin reductase, see 1.1.1, p. 18) seems to be of high importance. Thus, we may anticipate the relative separation of CK types in view of metabolism and functions. Table 11 also shows the differential degradation of free bases CKs manifested by the contrasting concentrations $8 \cdot 10^{-7}$ M and $4 \cdot 10^{-6}$ M as described earlier (see Figure 7B, p. 55).

Table 11: Profiles and concentrations (in pmol/gFW) of endogenous CKs after tZ application (the profiles are presented based on the nature of the N^6-side chain of CKs distinguishing tZ-, DZ-, cZ- and iP-types)

Concentration of applied *tZ* [M]	Endogenous CK levels [pmol/gFW]				
	tZ-type	DZ-type	*cZ*-type	iP-type	total CK pool
$3.2 \cdot 10^{-8}$	15.8	7.1	191.9	4.6	219
$1.6 \cdot 10^{-7}$	26.8	7.2	218.8	3.3	256
$8.0 \cdot 10^{-7}$	36.6	21.0	151.3	12.4	221
$4.0 \cdot 10^{-6}$	114.4	177.2	259.5	19.6	571
$2.0 \cdot 10^{-5}$	1 037.9	663.8	257.2	19.0	1 978
$1.0 \cdot 10^{-4}$	7 624.8	934.7	254.7	7.0	8 821
$5.0 \cdot 10^{-4}$	49 558.2	6 756.2	480.5	41.7	56 837
Fresh blank	37.2	8.4	246.8	33.1	325
Cultivated blank	15.1	11.1	283.4	210.7	520

At this point, the exclusive *tZ* and *tZ*9G influence in chlorophyll retention might be linked to the high *tZ*-type CKs content and especially to the elevated amount of free bases (see Table 10, p. 56) in oat cultivar Patrik.

The effect of exogenous iP application on the CK profiles was less pronounced than shown above for *tZ*-type CKs. The iP levels were not significantly elevated upon the cultivation in iP at lower than $1 \cdot 10^{-4}$ M concentration. The observed response to iP cultivation

can hardly be fitted by power regression model (R^2 = 0.6747, Figure 8A). Together with increased accumulation of iP-type CKs in cultivated oat leaf segments (see Table 13, p. 59), the lack of fit (Figure 8A) may indicate the iP role in dark senescence. Out of other CK substances, the levels of *tZ* (Figure 8B), *cZ*9G, iPR, iP9G and iPRMP were increased upon iP application in a dose-dependent manner (see Table 12, p. 59).

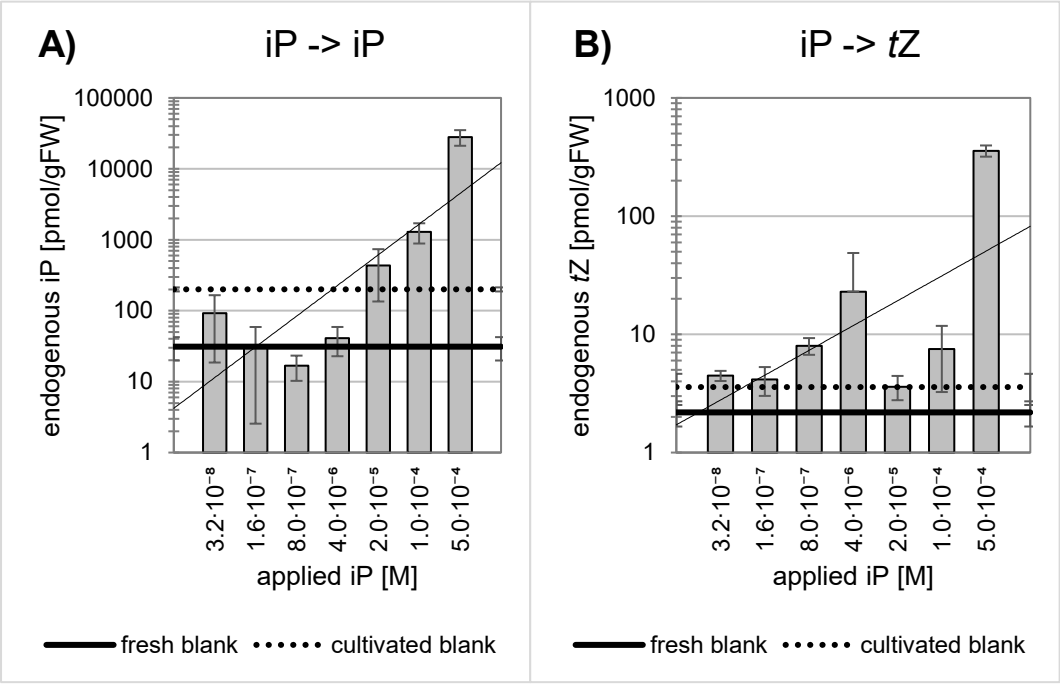


Figure 8: Exogenous iP application results in elevated iP levels (A) and tZ levels (B). Both trends are less pronounced and significant compared to the experiments in tZ-type CKs (A: $y = 499\,601 \cdot c^{0.6193}$, $R^2 = 0.6747$; B: $496.45 \cdot c^{0.3004}$, $R^2 = 0.4125$).

The application of iP7G resulted in an increase in endogenous levels of *tZ*9G and *cZ*7G. In analogy to *tZ*-glucosides, iP9G shows increased metabolization than iP7G. As a result of iP9G treatment, *tZ*9G, *cZ*9G, iP7G levels were elevated (see Table 12, p. 59). However, these endogenous CK content changes haven't influenced chlorophyll retention (as demonstrated in Figure 5, p. 50).

Table 12: Major changes in endogenous CK profiles after exogenous iP, iP7G and iP9G application

Applied cytokinin ($5 \cdot 10^{-4}$ M)	Triggered change of endogenous cytokinin level	Detected metabolite content [pmol/gFW]	Fold-change compared to cultivated blank (if detected in cultivated blank)	Coefficient of determination for power regression
iP	*tZ*	357	100	0.4125
	*cZ*9G	112	176	0.6930
	iP	28 128	141	0.6747
	iPR	186	———	0.9676
	iP9G	11 144	1 921	0.9698
	iPRMP	1 053	1 483	0.7026
iP7G	*tZ*9G	226	294	0.7830
	*cZ*7G	73	385	0.8958
	iP7G	387 715	111 733	0.9897
iP9G	*tZ*9G	153	157	0.7518
	*cZ*9G	794	1 241	0.9198
	iP7G	613	152	0.8676
	iP9G	103 005	17 759	0.9920

Table 13: Profiles and concentrations (in pmol/gFW) of endogenous CKs after iP application (the profiles are presented based on the nature of the N^6-side chain of CKs distinguishing tZ-, DZ-, cZ- and iP-types)

Concentration of applied iP [M]	Endogenous CK levels [pmol/gFW]				
	tZ-type	DZ-type	*cZ*-type	iP-type	total CK pool
$3.2 \cdot 10^{-8}$	7.4	15.0	214.3	96.5	333
$1.6 \cdot 10^{-7}$	10.9	6.7	295.1	39.4	352
$8.0 \cdot 10^{-7}$	15.7	13.5	272.5	29.6	331
$4.0 \cdot 10^{-6}$	39.3	15.2	263.8	99.9	418
$2.0 \cdot 10^{-5}$	246.9	20.1	252.5	1 244.1	1 764
$1.0 \cdot 10^{-4}$	16.8	22.7	282.9	1 943.0	2 265
$5.0 \cdot 10^{-4}$	483.2	137.1	950.1	40 510.9	42 081
Fresh blank	37.2	8.4	246.8	33.1	325
Cultivated blank	15.1	11.1	283.4	210.7	520

While the exogenous application of *tZ* and its glucosides has not largely influenced the iP-type CKs (see Table 10, p. 56), the application of iP and its glucosides influenced also *tZ*-type CKs (see Table 13, p. 59). Further research is needed to explain this phenomenon – the cytochrome P450 enzymes mediating iP conversion to *tZ* is supposed to be expressed predominantly in roots (see chapter 1.1.1, p. 18).

4.2 Effect of exogenously applied cytokinins on *Arabidopsis thaliana* seedlings

4.2.1 Alteration in the root system

Root system is very plastic and responds to numerous factors. Despite our effort to standardize growth conditions, we observed enormous variability in both primary root length and root architecture. In contrast to prevalent allorhizic root system (manifested by dominant primary root and lateral roots), variations analogous to homorhizic systems were common (see Figure 9A and B, p. 61).

These variations manifest shortened primary root length in control seedlings detectable as outliers by Grubbs test. As such, these outliers were excluded from further research. Besides altered root architecture, development of lateral and adventitious roots affects the primary root measurement. At the later sampling (14 DAS – days after sowing), the growth process becomes less quantifiable by primary root measurement as shown in Figure 10, p. 62. Thus, only data obtained in 11 DAS seedlings will be discussed here (even though the gene expression analysis was performed on 14 DAS seedlings).

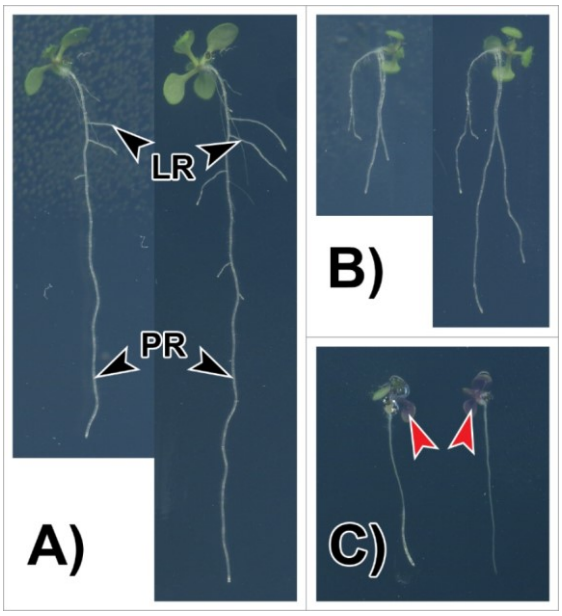


Figure 9: Observed Arabidopsis phenotypes

In the blocks A) and B) different phenotypes of control Arabidopsis seedlings at 11th (left) and 14th (right) day after sowing (DAS) are shown. Typically, Arabidopsis root architecture is allorhizic – dominant primary root (labelled as PR) produces lateral roots (LR), as seen at A). On the other hand, variations analogous to homorhizic systems were observed (B). C) Phenotype modified by tZ addition to cultivation medium in 14 DAS seedlings. Primary roots are shortened and lateral root development is delayed. Shoots respond to the changes in root architecture by retarded growth and discoloration (red arrows).

The variability in observed data for control plants included also significant differences (unpaired two-tailed Student's t-test, p-value < 0.001) between measurements at seedlings sown on several dates (see Figure 11, p. 62) – the average primary root length 11 DAS in plants sown on 11.11.2016 was 3.99 cm compared to 3.36 cm and 3.19 cm of those sown on 26.05.2017, and 28.07.2017 respectively. To overcome this obstacle in CK effect comparison (experiments for tested substances were held on different dates), measured primary root lengths are shown relatively to the respective control.

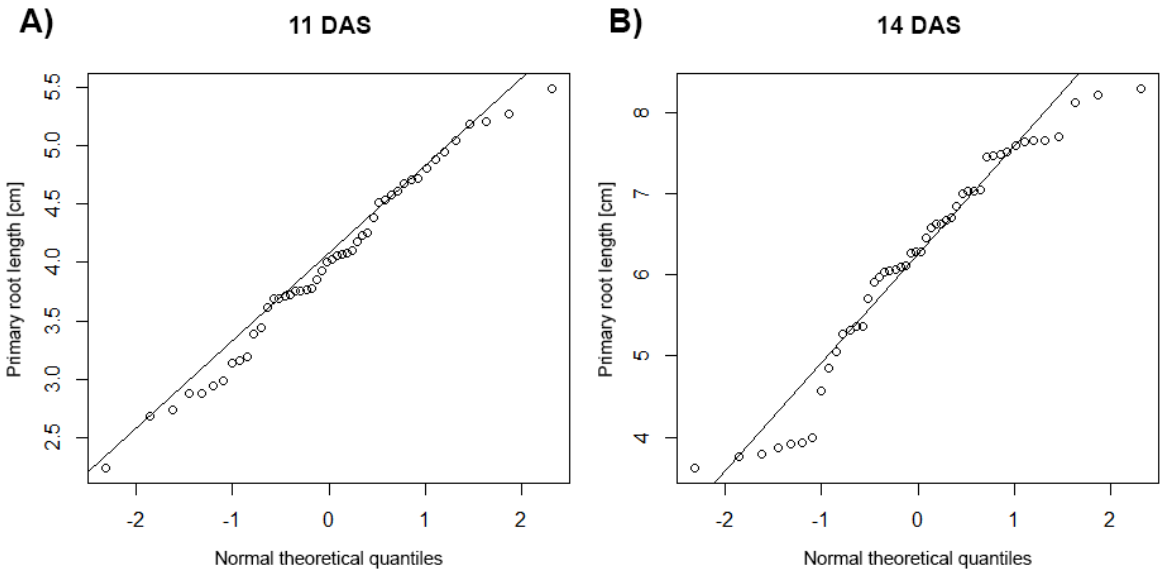


Figure 10: Quantile - quantile plots comparing the measured primary root length of control Arabidopsis plants sown on 11. 11. 2016 to normal distribution

A) Data measured on 11 day after sowing (DAS), average primary root length 3.99 cm, Shapiro-Wilk normality test p-value is 0.7396. B) Data measured on 14 DAS, average primary root length 6.19 cm, Shapiro-Wilk normality test p-value 0.01926.

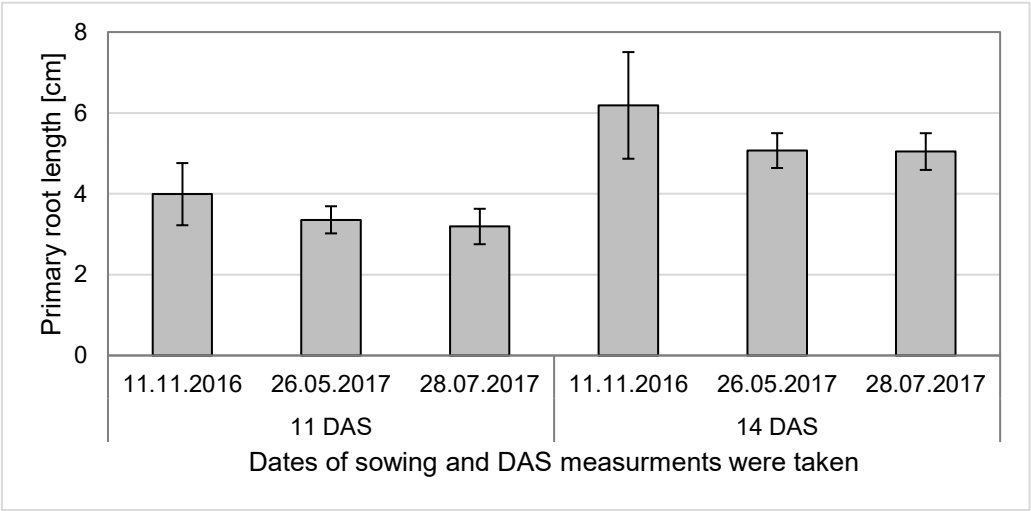


Figure 11: Variability of control Arabidopsis primary root length with respect to seedlings age and the date of sowing

Plant material used for this experiment was sown on three dates (11. 11. 2016, 26. 05. 2017, and 28.07.2017). For each variant, the primary root length was measured on 11 DAS (days after sowing) and 14 DAS respectively. Error bars represent the standard deviation ($n \geq 40$).

Exogenous application of CKs resulted in shortening of Arabidopsis primary root length, which holds especially for free bases (i.e. *tZ* and iP) as visualized in Figure 12, p. 63. While the *tZ* significantly reduced primary root length (to 34 % of control length on average), its *N7*- and *N9*-glucosides had almost no effect (the average primary root length was 98 % and 99 % of control, respectively; Figure 12A, p. 63). On the other hand, supplementation of media with iP and its *N*-glucosides resulted in primary root growth retardation (Figure 12B, p. 63). Whereas iP ($5 \cdot 10^{-7}$ M) caused reduction of primary root to 55 % of control, iP7G and iP9G were less effective at the same concentration (70 and 71 % root reduction, respectively) compared to the higher one ($5 \cdot 10^{-4}$ M; 56 and 54 % root reduction, respectively). Additionally to the effect of exogenously applied CKs on root growth, shoot alterations including shoot growth retardation and discolouration were recorded as well (see Figure 9, p. 61).

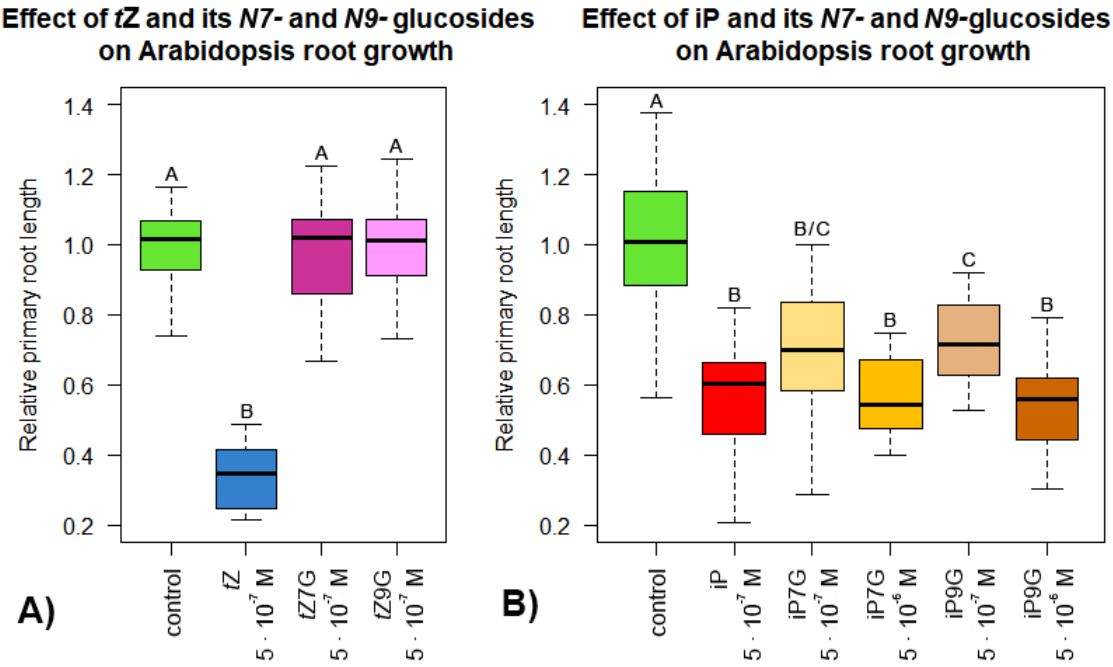


Figure 12: Cytokinin effect on Arabidopsis primary root length in 11 days after sowing seedlings

On the left, the effects of tZ and its glucosides (tZ7G and tZ9G) are shown (A), on right, the same for iP (B). Letters above boxplots indicate different groups according to the Tukey multiple comparison test.

4.2.2 RNA isolation

RNA from both shoot and root Arabidopsis segments was isolated to assess the effect of exogenously applied CKs (*tZ*, iP and their glucosides) on Arabidopsis expression profiles. The RNA isolation step was omitted in root samples with phenotype severely affected by exogenous CK application (plants grown on media enriched with $5 \cdot 10^{-7}$ M *tZ*) due to the low amount of material available resulting from the growth retardation. Where possible, more biological replicates were taken. Content and quality of RNA isolated from processed samples measured with NanoDrop One[c] are demonstrated in Table 14.

Table 14: Content and quality of RNA isolated from processed samples

Sample nr.	Sample identification	Total RNA		Total RNA after DNase treatment	
		Contaminants detected	RNA content [ng/µl]	Contaminants detected	RNA content [ng/µl]
1	control shoots	none	1158.7	none	923.7
2	control shoots	none	1733.6	none	1248.9
3	iP9G ($5 \cdot 10^{-6}$ M) shoots	none	442.7	none	350.8
4	iP9G ($5 \cdot 10^{-7}$ M) shoots	none	833.7	none	662.4
5	iP7G ($5 \cdot 10^{-6}$ M) shoots	none	493.5	none	394.5
6	iP7G ($5 \cdot 10^{-7}$ M) shoots	none	531.7	none	414.5
7	control shoots	none	1389.9	none	697.4
8	control shoots	none	1102.2	none	812.8
9	*tZ* ($5 \cdot 10^{-7}$ M) shoots	none	480.5	none	361.0
10	*tZ*7G ($5 \cdot 10^{-7}$ M) shoots	none	625.7	none	475.1
11	*tZ*7G ($5 \cdot 10^{-7}$ M) shoots	none	302.4	none	232.2
12	*tZ*7G ($5 \cdot 10^{-7}$ M) shoots	none	561.3	guanidine	451.3
13	*tZ*9G ($5 \cdot 10^{-7}$ M) shoots	none	614.6	none	481.8
14	*tZ*9G ($5 \cdot 10^{-7}$ M) shoots	guanidine	256.3	guanidine	200.8
15	control roots	none	298.6	none	214.0
16	iP9G ($5 \cdot 10^{-6}$ M) roots	guanidine	105.7	guanidine	77.3
17	iP9G ($5 \cdot 10^{-7}$ M) roots	none	98.7	none	85.1
18	iP7G ($5 \cdot 10^{-6}$ M) roots	none	118.0	none	94.3
19	iP7G ($5 \cdot 10^{-7}$ M) roots	guanidine	271.8	guanidine	206.4
20	control roots	guanidine	122.8	guanidine	95.7
21	*tZ*7G ($5 \cdot 10^{-7}$ M) roots	guanidine	98.3	guanidine	73.6
22	*tZ*9G ($5 \cdot 10^{-7}$ M) roots	guanidine	72.9	guanidine	55.6
23	iP ($5 \cdot 10^{-7}$ M) roots	none	63.4	none	48.2
24	iP ($5 \cdot 10^{-7}$ M) shoots	none	8.7	———	——

In Table 14, p. 64 the resulting values after DNase treatment are shown. Due to the low RNA content, sample 24 was excluded from further investigations (i.e. shoots grown on media enriched with $5 \cdot 10^{-7}$ M iP).

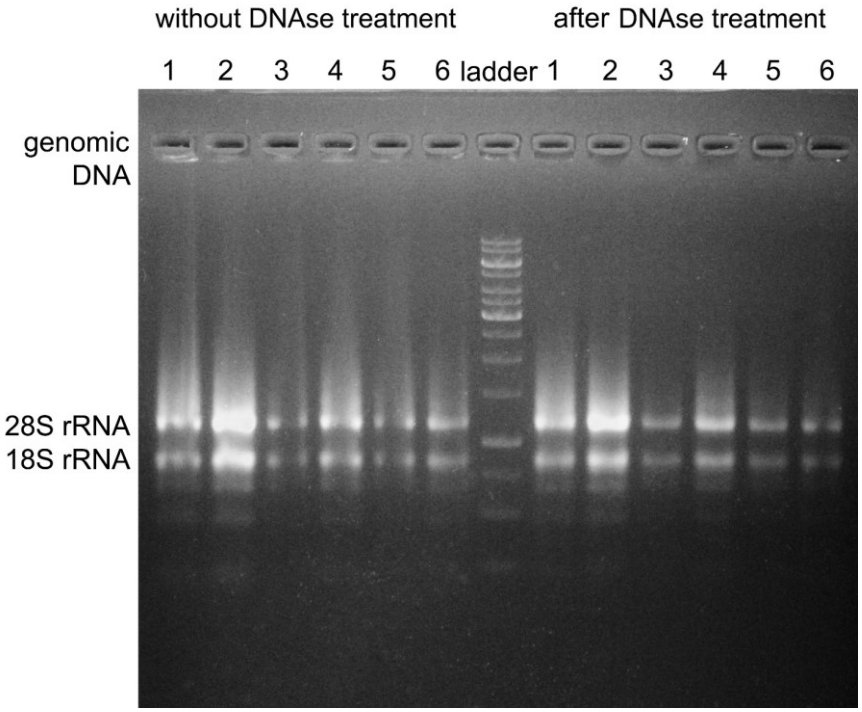


Figure 13: Visualization of representative RNA selected samples before and after DNase treatment

Strong bands corresponding 18S and 28S rRNA are visible in all selected samples. Thus, isolated RNA is relatively intact. Note the upper band in samples without DNase treatment that proves successful removal of the genomic DNA contamination in purification step.

Samples nr. 1 – 2 are control shoots; 3 – 4 are shoots grown on iP9G enriched media concentrated $5 \cdot 10^{-6}$ M and $5 \cdot 10^{-7}$ M, respectively; 5 – 6 are similarly shoots grown on iP7G enriched media concentrated $5 \cdot 10^{-6}$ M and $5 \cdot 10^{-7}$ M.

4.2.3 Expression profiles

In Arabidopsis shoots and roots, the response of various CK-related genes on CK (*tZ*, iP, and their glucosides) treatments was evaluated. Genes of interest included those encoding CK biosynthetic enzymes (IPT5 and IPT9), glucosylation enzymes (UGT76C1, UGT76C2,

and UGT85A1), CK transporter (ABCG14), and mediators of CK signalling (CK receptor AHK4 and transcription factors ARR5, ARR7, ARR16, and CRF5).

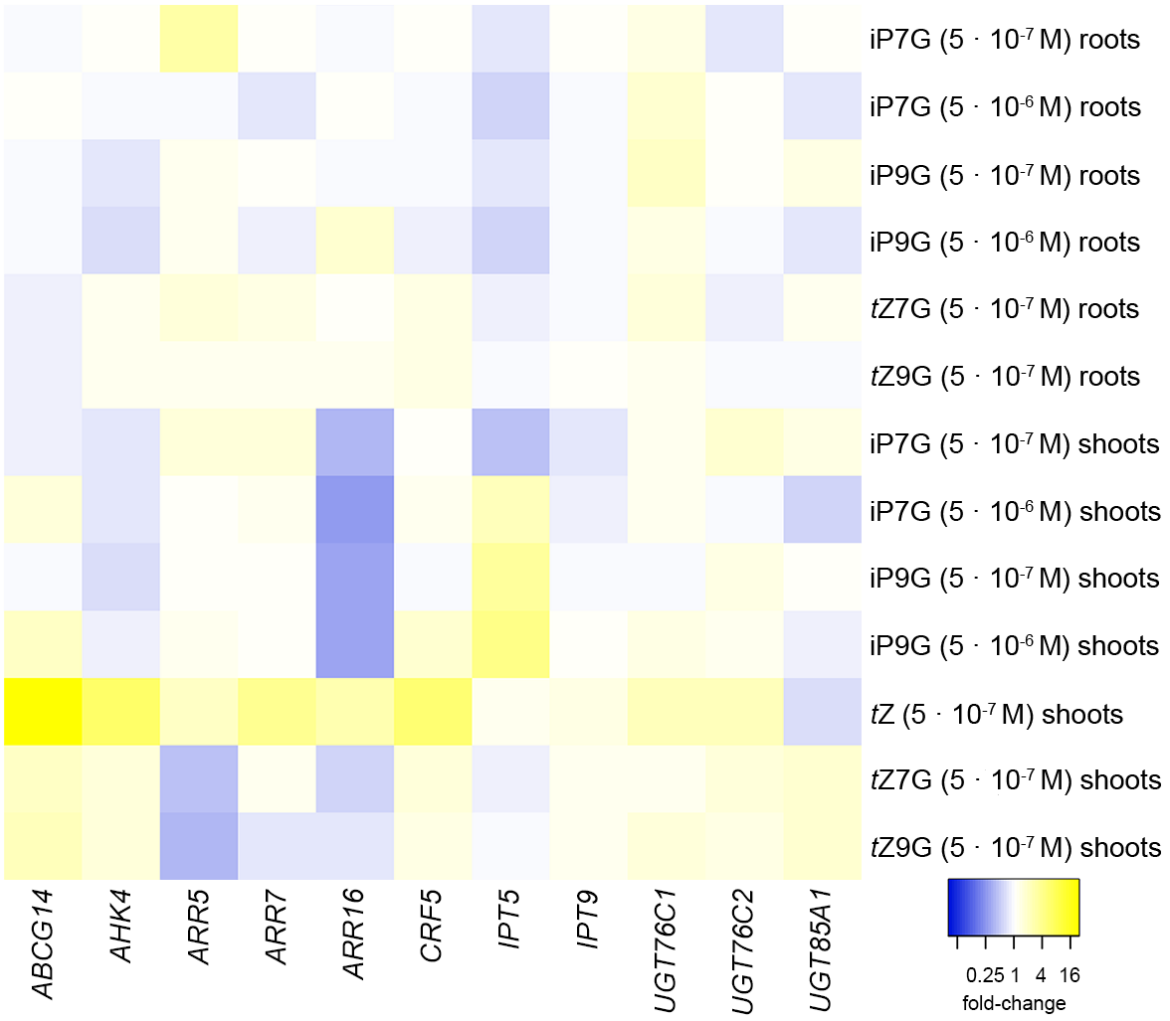


Figure 14: Expression heatmap

The expression of selected genes was quantified by the comparison to expression of two housekeeping genes (ACT2 and EF1ALPHA). For the heatmap preparation, data were normalized to the control samples and expressed fold-change. Created with [161].

Changes in the gene expression were mostly rather mild as demonstrated in Figure 14. Out of the tested CKs, the highest effect on the expression of the observed gene set was revealed for *tZ*, however, this CK was tested only on shoot samples (as root growth was reduced to the extent making their sampling impossible). In Arabidopsis shoots grown on *tZ*-containing medium, enhanced expression profiles of several CK genes were detected.

The strongest upregulation compared to control was found for the CK transporter ABCG14 (24.4-fold, SD 0.47) followed by AHK4 (6.52-fold, SD 0.36), CRF5 (5.60-fold, SD 0.13), and ARR7 (3.91-fold, SD 0.13) while the transcripts abundance of UGT85A1 was slightly downregulated (0.61-fold, SD 0.15).

The relative expression profiles after application of *tZ*7G and *tZ*9G in Arabidopsis shoots resembled those after *tZ* treatment in terms of higher relative gene expression levels of *ABCG14*, *CRF5* (about 2-fold higher, SD around 0.20), and *AHK4* (approximately 1.6-fold, SD 0.2). Contrary to *tZ*, downregulated gene transcription profiles were recorded for *ARR5* (0.40-fold, SD 0.15 for *tZ*9G and 0.43, SD 0.30 for *tZ*7G) and *ARR16* (0.75-fold, SD 0.14 for *tZ*9G and 0.54, SD 0.19 for *tZ*7G). Analogically, UGT85A1 expression was higher (on average 1.8-fold, SD 0.26) for both *tZ*7G and *tZ*9G treatments whereas *tZ* alone caused its decreased expression (to 0.61-fold, SD 0.15).

Unfortunately, no iP treated samples contained enough specific RNA transcript to perform q-RT PCR. On the other hand, the effects of iP7G and iP9G were tested in two concentrations ($5 \cdot 10^{-7}$ M and $5 \cdot 10^{-6}$ M). Observed expression effects of the two concentrations of the tested iP-glucosides were in some instances contradictive, probably due to high variability. For example, the IPT5 relative expression was downregulated (0.44-fold, SD 0.7) compared to control in shoots grown on $5 \cdot 10^{-7}$ M iP7G medium, whereas 2.2-fold upregulation was found on $5 \cdot 10^{-6}$ M iP7G (SD 0.47).

Besides the above-mentioned changes in IPT5 expression in response to iP7G treatment, also iP9G affected IPT5 transcription levels. The detected upregulation by iP9G was 4.42-fold ($5 \cdot 10^{-7}$ M, SD 0.25) and 3.28-fold ($5 . 10^{-6}$ M, SD 0.13). The genes downregulated in iP7G treated seedlings were *ARR16* (0.25-fold change, SD 0.16 for higher concentrated

medium), *AHK4* (analogically 0.73-fold change, SD 0.17), and *IPT9* (0.78-fold change, SD 0.21). After iP9G treatment, *ARR16* and *AHK4* were also downregulated in shoots, although *AHK4* only in case of lower, i.e. $5 \cdot 10^{-7}$ M, iP9G concentration.

The root expression profiles widely differed from those of shoots. The ARR16, whose shoot expression was decreased in plants grown on both iP and *tZ*-glucosides containing media, was upregulated in roots treated with $5 \cdot 10^{-6}$ iP9G (1.82-fold, SD 0.68). Although the changes in expression levels were, in general, less pronounced and significant in roots, 3.15-fold ARR5 upregulation (SD 1.29) following $5 \cdot 10^{-7}$ M iP7G treatment and 1.9-fold UGT76C1 upregulation (SD 0.52) following $5 \cdot 10^{-7}$ M iP9G treatment should be mentioned.

5 Discussion

Amongst the CK derivatives, *N*-glucosides are generally considered irreversible products lacking physiological activity [44]. The enzymes catalysing *N*-glucosylation (uridine diphosphate-glycosyltransferases UGT76C1 and UGT76C2) were shown to convert CK active forms (free bases) to putatively inactive *N*-glucosides and thus to control efficiently the CK homeostasis in plants [69]. Recently, the evidence of a distinguished role of CK *N*-glucosides in plants has been accumulated. In addition to the observed activity of *N9*- glucosides in a few bioassays, the *N9*-glycosylic bond was shown to undergo *in vitro* enzymatic cleavage by CKX or hydrolysis by β-glucosidase in maize [162, 163]. Even though CK metabolism is perceived as largely characterized, numerous blank spots still occur [164]. Whereas a substantial amount of knowledge regards Arabidopsis, much less is known of the plants with larger genomes that have not been read (e.g. oat). That's why a considerable part of this diploma work relates to experiments on this plant species.

5.1 The cytokinin effect on oat leaf senescence

Chlorophyll retention in senescing leaves is a well-known effect of CKs, documented both when exogenously supplied [24], as well as in transgenic plants with elevated endogenous CK levels [165, 166]. While there are literature data regarding *tZ* and iP effects in the chlorophyll retention bioassay of detached oat leaves [135, 137], the impact of their glucosides has been rather unstudied in the respective model (for comparison, see [140, 167, 168]). In our previous study made on the leaf segments of different oat cultivar (cv. Abel) from that used here, we showed senescence deceleration after exogenous *tZ*9G application (similar to those of *tZ*), but no such effect was observed for *tZ*7G. Despite a widely accepted view of *tZ*9G as irreversibly deactivated CK metabolite, transglucosylation to *O*-glucosides (representing prevalent CK forms in fresh oat leaves) was suggested as a possible cause

of *t*Z9G activity in this bioassay [134]. The inability of iP-type CKs to retain the leaf chlorophyll observed earlier [135, 137] is in accordance with this opinion. To test our hypothesis, a similar set of experiments was performed for both *t*Z and iP and their *N*-glucosides on another oat cultivar, cv. Partik.

The chlorophyll retention bioassay on oat (cv. Patrik) leaf segments confirmed our previous findings documenting *t*Z9G activity and, in accordance with the literature data [135, 137], also showed inactivity of iP (and moreover also inactivity of iP-glucosides). The endogenous CK profiling, however, did not confirm the increase of *c*ZOG levels induced by exogenous *t*Z9G application demonstrated in our previous study [134], and thus questioned the hypothesized transglucosylation to *O*-glucosides as a potential reason of *t*Z9G activity in this system. Rather surprisingly, the *c*ZOG levels remained almost unchanged throughout the experimental settings and none of the applied substances (*t*Z, iP and their *N*-glucosides) nor the cultivation alone significantly affected the *c*ZOG levels (data not shown).

On the other hand, in agreement with our previous work, the *t*Z9G application induced a considerable increase of endogenous *t*Z levels (see Table 10, p. 56). This effect was, however, not observed for *t*Z7G treatment, which might satisfactorily explain the difference between the two substances. Even though we observed also the enhancement of endogenous *t*Z levels in the leaf segments incubated in iP solutions, the outcome was not concentration-dependent and thus easily interpretable (see Figure 8B, p. 58). The formation of *t*Z following the iP treatment might be related to the *de novo* biosynthesis or to the impact of cytochrome P450 enzymes. These actions are presumably slower than the metabolic conversion observed after exogenous *t*Z9G application (while *t*Z9G affected chlorophyll retention, no effect was observed after iP treatment; potentially unknown signalling role of *t*Z9G is less probable).

An overall trend observed after exogenous application of selected substances (*tZ*, iP and their *N*-glucosides) was their accumulation in leaf segments as manifested by elevated total CK levels (much higher for particular *N*-glucosides compared to free bases; see Table 9, p. 51). While CK free bases may be easily degraded by oxidative cleavage by CKX e.g. [44, 64], their corresponding *N*-glucosides are generally considered to be resistant against the CKX attack. Although a few exceptions to this generalization exist (recombinant AtCKX [169, 170], barley HvCKX [171] and maize ZmCKX [63] enzyme isoforms showing ability to degrade CK-*N9*-glucosides as well), different CKX affinity may explain differences in the total CK levels in segments treated with free bases compared to those subjected to *N*-glucosides.

Interestingly, not only *tZ*9G seems to possess different properties and to exhibit distinct functions compared to *tZ*7G. Observed anomaly in enormous iP7G accumulation in oat leaves segments exceeding that of other *N*-glucosides might represent another piece of puzzle in oat metabolism (see Table 9, p. 51). The extremely high iP7G content could be related to its storage function [67] and thus possibly to its weak metabolization (see Table 12, p. 59).

The explanation for observed inconsistencies from our previous work [134] might consist of the use of a different oat cultivar or include various condition interplay [172]. Despite the simplification of the experimental model (detached leaves in controlled environment), plant tissue remains highly organized, representing a complex system susceptible to both exogenous and intrinsic factors. Exogenously applied CKs might differ in their tissue penetration, signalling response [173] and metabolic conversion or degradation [35]. Moreover, the endogenous CK profiles might be affected also by tissue adaptation [111]. In contribution to CK induced changes, the interaction with other plant hormone networks

(crosstalk) should be considered as well [174]. The outcomes of several experiments resulted in non-monotonic dose-response curves [175] (e.g. markedly reduced chlorophyll retention after application of $5 \cdot 10^{-4}$ M *tZ* and *tZ*9G solutions compared to $1 \cdot 10^{-4}$ M concentrated respective solutions, see Figure 5, p. 50). Indeed, high concentrated CK solutions induced contradictive reaction such as e.g. the leaf segments necrosis in the basal parts (mentioned in part 4.1.1, p. 49), which has been partially characterized in literature [176, 177]. Additionally, the data analysis is even more complicated due to the differential activity of particular CK forms with respect to their (extra)cellular localization [178].

5.2 Arabidopsis growth on CK enriched media

Changes in morphogenesis of the root system in response to exogenous CKs represent a well-known phenomenon originally revealed as early as in 1959 in pea [179]. Despite extensive research of the CK involvement in the root formation and growth in the subsequent years [144-147], the underlying mechanisms haven't been discovered until molecular biology studies carried out almost fifty years later [148, 180-182].

In contrast to the oat leaf senescence bioassay, not only *tZ*- but also iP-type CKs were found here to affect considerably the Arabidopsis root growth. It is known that the exogenous *tZ* application inhibits cell proliferation [182] and auxin flow [149]. It might be also involved in the root-to-shoot signalling, as CKs obviously represent long-distance signal molecules mediating the shoot-root communication. This role could explain the root susceptibility to exogenous iP-type CKs that possess mock signals of shoot [183].

The highest effect on the main root length was found for *tZ* (see Figure 12A, p. 63). Also, other phenotypic traits such as e.g. anthocyanin accumulation in the shoots were recorded (see Figure 9, p. 61) [184]. Although the iP treatment was less potent compared to *tZ*, iP

still significantly affected the Arabidopsis root system being about twice as active as iP7G and iP9G applied at the same concentrations and exhibiting a comparable activity as corresponding *N*-glucosides used in 10-fold excess (Figure 12B, p. 63). Interestingly, no effects on the primary root length were found for *tZ*-glucosides (Figure 12A, p. 63).

Significant effects of applied CKs on Arabidopsis phenotype were, however, not clearly supported by the expression data. For instance, whereas *tZ* induced the most pronounced changes of phenotype, the average difference in the expression of selected genes of CK metabolism, transport and signalling as compared to the control was 3-fold. This might be perceived as another manifestation of the complexity of the hormonal control. Below, the differences in expression of the selected genes with respect to their physiological function are discussed.

Function of the ABCG14 transporter consists of CK loading to the xylem [131]. The production of *tZ*-type CKs is located in roots and the ABCG14 mediates their transport to shoots, which represents a crucial long-distance signal for the shoot development [183]. Thus, it is not surprising that the exogenous application of *tZ* enhances *ABCG14* expression. The same effect was recorded, although in lesser extent, also for *tZ*7G and *tZ*9G (especially for shoots; Figure 14, p. 66). One may thus anticipate that the upregulation of the transport system leads to the phenotype severity observed in plants sown on *tZ* enriched medium (moreover the ABCG14 is supposed to be expressed in this developmental stage primarily in roots and hypocotyl, not in shoots as reported in [185]). The effects of iP-glucosides on the *ABCG14* expression were found rather contradictory depending on the concentration of the applied substance and the type of the tissue (shoots *vs.* roots).

Interestingly, relative expression of one of the CK receptors, AHK4, was predominantly decreased by the application of iP-glucosides, while *tZ*-type CKs (both *tZ* and its glucosides) increased it substantially. This differential reaction is remarkable especially due to the fact that the protein affinity to both CK-types was reported approximately equal [89]. This expression changes might fine-tune the CK signalling and thus help to cope with exogenous CK supply. With this respect, adding the gene expression analysis also for AHK3, characteristic by its strong *tZ* binding preference, is highly desirable.

All of the ARRs studied here belong to the type-A ARR family. The type-A *ARR* genes, whose transcription can be rapidly induced by CKs, act as negative regulators of CK signalling [116]. From this point of view, their observed shoot upregulation caused by *tZ* and contrarily downregulation caused by its glucosides (both *t*Z7G and *tZ*9G) seem intriguing. Application of iP7G and iP9G slightly increased the relative expression of *ARR5* and *ARR7* genes in shoots, which was in contrast to a relatively strong downregulation of *ARR16* by the two iP-glucosides. The data concerning effects of *tZ*- and iP-glucosides on the expression of *ARR*s in roots seem to be rather ambiguous. Although little is still known about the biochemistry of the type-A ARR protein activity, our data contribute to the knowledge of regulatory mechanisms of CK signalling. In this regard, the CK profiling could provide another important piece of information – it might be just a coincidence that iP7G treatment affects both phenotype and *ARR16* expression in Arabidopsis, whereas in oat leaves no such effect was observed while iP7G was strongly accumulated.

The CK biosynthesis in Arabidopsis seedlings seems to be somehow affected by exogenous CK application, as indicated by differential *IPT5* expression. Although the data demonstrating the *IPT5* expression in shoots are rather inconsistent, the upregulation by iP9G is obvious. Interestingly, a counteractive effect of the two iP7G concentrations

on the *IPT5* expression in shoots was observed. The applied *N*-glucosides of *tZ* and iP exhibited only rather mild or none effects on the relative expression of both *IPT5* and *IPT9* genes.

In Arabidopsis, only three UGT genes are known to deactivate CKs *in vivo*, UGT76C1, UGT76C2 and UGT85A1. In the literature, *UGT85A1* was found to be sensitive to *tZ* treatment which increased its expression mildly [67]. By contrast, in our work we found a relatively strong shoot downregulation of *UGT85A1* expression by *tZ*, which might be due to the different age of the plants and/or the distinct concentrations of the applied CKs. On the other hand, positive effects on *UGT85A1* expression were revealed for *tZ*7G and *tZ*9G in shoots as well as in roots. Relative expressions of both *N*-specific *UGT*s, i.e. *UGT76C1* and *UGT76C2*, were mostly mildly upregulated in roots by the treatments with tested CKs in this study, however, a few exceptions to this generalization exist for *UGT76C2*. In shoots, the effects of applied CKs on the *UGT76C1* and *UGT76C2* expression is hard to generalize at all.

In spite of the fact that the differences in expression of the selected genes after application of tested CKs are in some cases rather inconsistent, the results presented here provide a considerable and beneficial contribution for the insight into the regulatory mechanisms of CK metabolism, signalling and transport.

6 Conclusions

The effect of exogenously applied cytokinins (CKs) including *tZ*, iP and their *N*- glucosides have been studied on oat (*Avena sativa* L.) cv. Patrik senescence and *Arabidopsis thaliana* (L.) Heynh root growth and gene expression. Following findings belong to the most important ones:

1) Both *trans*-zeatin (*tZ*) and *tZ N9*-glucoside (*tZ*9G) retard detached oat leaf senescence in darkness. No effect was observed for *tZ N7*-glucoside (*tZ*7G), N^6-(Δ^2-isopentenyl adenine) (iP), iP*N7*-glucoside (iP7G) and iP*N9*-glucoside (iP9G).
2) The metabolization of *N7*- and *N9*- CK glucosides in oat leaf segments significantly differs. The CK profiling showed higher metabolization of *N9*-glucosides which includes also formation of free-bases CK forms. Contrary, the *N7*-glucosides were rather accumulated. The hypothesized lack of activity of iP-type CKs in the respective model was confirmed but remains to be elucidated.
3) The Arabidopsis primary root growth retardation was recorded on media containing *tZ* and iP, and, to a lesser extent, also on media enriched with iP7G and iP9G. No effect was observed for *tZ*7G and *tZ*9G which might refer to missing long-distance signalling role of *tZ*-glucosides compared to free-base.
4) Performed transcription analysis of Arabidopsis grown on CK supplied media revealed several differentially expressed genes. With regards to phenotype severity, the role of ABCG14, ARR5, and ARR16 should be further investigated.

Although we did not fully confirm our previous hypothesis [134], data in this diploma work represent a considerable contribution to the current research of CKs. They partially argue

against the established notion of CK *N*-glucosides, at least those glycosylated at *N9*-position, as deactivation and/or metabolically stable CK forms, suggest their possibly higher relevance to CK biology than previously thought and indicate that a general image of CK *N*-glucosides as inactive and irreversible compounds should be reconsidered.

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