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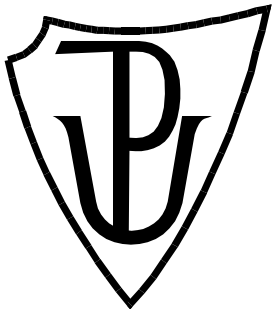

Subcellular localization of maize cytokinin dehydrogenases

DIPLOMA THESIS

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Study branch:	Biochemistry
Study mode:	Full-time
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Submitted:	7th May

I hereby declare that this diploma thesis has been written solely by me. All the sources cited in this work are listed in the reference part.

Olomouc, 7th May 2015 ----------

Acknowledgement

Foremost, I would like to express my sincere gratitude to my supervisor Mgr. David Zalabák, Ph.D. for the continuous support of my diploma thesis and laboratory work, for his patience, motivation, enthusiasm, and his precious advices. His guidance helped me in all the time of my work and writing of this thesis. Besides my supervisor, I would like to thank all members of Department of Molecular Biology for their encouragement, help during my laboratory work and insightful comments. I would like to thank doc. Mgr. Petr Galuszka, Ph.D. for offering me diverse internship opportunities and leading me working on many exciting projects. My sincere thanks also goes to prof. RNDr. Jozef Šamaj, DrSc. and Mgr. Olga Šamajová, Dr. for offering me to use microscopic equipment of their Department of Cell Biology. Special thanks belongs to RNDr. Ondřej Plíhal, Ph.D. for his help with microscope image processing and his students Mgr. Karolína Kubiasová and Bc. Karolína Šenková, who encouraged me during my work, helped me everytime I needed and for all the fun we have had in the last two years.

Last but not the least, I would like to thank my family, my friends and my partner for the unceasing encouragement, support and attention all the time I worked on my diploma thesis.

Bibliografická identifikace

Jméno a příjmení autora	Bc. Patricie Johnová
Název práce	Subcelulární lokalizace kukuřičných cytokinindehydrogenas
Typ práce	Diplomová
Pracoviště	Oddělení molekulární biologie
Vedoucí práce	Mgr. David Zalabák, Ph.D.
Rok obhajoby práce	2015

Abstrakt

Cytokinindehydrogenasy (CKX; EC 1.5.99.12) patří do významné třídy enzymů, které zprostředkovávají irreversibilní degradaci rostlinných hormonů cytokininů a přispívají tak k udržování jejich rovnováhy. V mnoha rostlinných druzích byly nalezeny malé genové rodiny *CKX*. Jedním z jedinečných aspektů CKX je jejich odlišná subcelulární lokalizace, která zřejmě úzce souvisí s jejich odlišnou substrátovou specifitu. Doposud byla nejlépe charakterizována rodina *AtCKX* modelové dvouděložné rostliny *Arabidopsis thaliana*, která čítá sedm členů. Bylo dokázáno, že odlišná subcelulární lokalizace spolu s odlišnou časovou distibucí CKX mají silný vliv na fenotyp rostlin overexprimujících *AtCKX*, což vede ke vzniku typického fenotypu z nedostatku cytokininů. Genom kukuřice zahrnuje celkem třináct genů *ZmCKX*, jejichž biochemické vlastnostni a expresní profil v jednotlivých pletivech byly pospsány teprve nedávno. Nicméne, informace o subcelulární lokalizaci jednotlivých CKX v jednoděložných rostlinách nejsou stále jasné. V předchozích studiích byly detailně charakterizovány pouze dvě isoformy ZmCKX, přičemž se ukázalo, že ZmCKX1 je sekretována do apoplastu a ZmCKX10 se vyskytuje v cytosolu a zřejmě i v jádře.

Cílem předkládané práce bylo zjistit subcellularní lokalizaci deseti isoenzymů ZmCKX. Jednotlivé *ZmCKX* (*1*, *2*, *4a*, *4b*, *5*, 6, *8*, *9*, *10* and *12*) byly fúzovány se zeleným fluorescenčním proteinem (GFP) a za použití laserové skenovací konfokální mikroskopie byla studována jejich lokalizace na subcelulární úrovni. Transietní expresí *Ubi1*::*ZmCKX*-*GFP* v protoplastech kukuřice bylo zjištěno, že většina ZmCKX je asociována s endoplasmatickým retikulem (ER). K potvrzení těchto výsledků byly odvozeny stabilní buněčné linie *Arabidopsis*, exprimující *CaMV35S*::*ZmCKX*-*GFP*. Téměř všechny isoformy ZmCKX byly překvapivě nalezeny ve vakuolách. Tyto výsledky poukazují na možnost, že lokalizace v ER je pouze přechodným bodem před vstupem ZmCKX do vakuol, které jsou zřejmě cílem většiny isoenzymů ZmCKX. Vedle toho byla v experimentálně potvrzena apoplastická lokalizace ZmCKX1 a cytosolární/jaderná lokalizace ZmCKX10. Tato práce jako první poskytla náhled na distribuci cytokininového katabolismu v jednoděložných rostlinách.

Klíčová slova	Buněčná kultura *Arabidopsis*, CKX, cytokinin, ER, GFP, kukuřice, subcelulární lokalizace, vakuola.
Počet stran	69
Počet příloh	0
Jazyk	Anglický

Bibliographical identification

Autor's first name and surname	Bc. Patricie Johnová
Title	Subcellular localization of maize cytokinin dehydrogenases
Type of thesis	Diploma
Department	Department of molecular biology
Supervisor	Mgr. David Zalabák, Ph.D.
The year of presentation	2015

Abstract

Cytokinin dehydrogenases (CKX; EC 1.5.99.12) belong to the important class of enzymes mediating irreversible degradation of plant hormones cytokinins and thus contribute to the maintainance of cytokinin homestasis. Presence of small CKX gene families have been reported in many plant species. One of the unique aspects of CKX isozymes is their diverse subcellular localization, which is also assumed to be responsible for preferences of CKX to various substrates. So far, best characterized is *AtCKX* gene family from dicotyledonous plant model *Arabidopsis thaliana*, comprising seven members. Overexpression of *AtCKX* genes proved that distinct subcellular localization along with the distinct temporal distribution of CKX have strong impact on plant phenotype, as a result of cytokinin-deficient syndrome. The maize genome encompasses thirteen *ZmCKX* members, whose biochemical properties as well as tissue expression patterns were described in detail only recently. However, the information on the CKX subcellular localization in monocots is still not clear. Previously only two isoforms has been studied in detail: ZmCKX1 which was shown to be secreted to apoplastic space and ZmCKX10 indicating dual expression pattern, partially in cytosol and nucleus.

The goal of the present study was to disclose subcellular distribution of ten ZmCKX isozymes. Individual *ZmCKX* (*1*, *2*, *4a*, *4b*, *5*, 6, *8*, *9*, *10* and *12*) genes were tagged to green fluorescent protein (GFP) and their expression pattern was examined using live cell confocal microscopy. Transient expression of *Ubi1*::*ZmCKX-GFP* in maize protoplasts, revealed the majority of ZmCKX isoforms is associated with endoplasmic reticulum (ER). In order to confirm these results stable cell lines of *Arabidopsis* expressing *CaMV35S::ZmCKX-GFP* were established. Surprisingly, almost all ZmCKXs were primarily targeted to vacuoles. These results refer to the possibility that ER-localization is a transition point to vacuoles, which seems to be the final destination of most of the ZmCKX isozymes. In addition, the localization of apoplastic isoform ZmCKX1, and cytosolic/nuclear ZmCKX10 was proved in both assays. This study provided the first insight into the spatial distribution of cytokinin catabolism in monocotyledonous species.

Keywords	*Arabidopsis* cell culture, CKX, cytokinin, ER, GFP, maize, subcellular localization, vacuole.
Number of pages	69
Number of appendices	0
Language	English

CONTENT

AIMS OF WORK

Theoretical part

Review on cytokinins and their metabolism, focused mainly on cytokinin dehydrogenases.

Experimental work

- Cloning of *CaMV35S::ZmCKX-GFP* constructs (in pGWB5 binary vector)
- Transient expression of *ZmCKX-GFP* fusion genes in maize protoplasts
- Stable expression of *ZmCKX-GFP* fusion genes in *Arabidopsis thaliana* L*er* cell suspension cultures
- Analysis of ZmCKX-GFP fusion proteins subcellular localization using laser scanning confocal microscopy
- Analysis of ZmCKX-GFP expression by Western blotting
- Measuring of ZmCKX-GFP enzymatic activity in stable *A.* lines

1 Introduction – plant hormones

The existence of substance participating at low concentration in regulation of growth was proved already in 19th century in the experiment of Dutch biologist Fritz Went. He demonstrated the effect of a substance, which promoted plant growth. This substance was later identified as indole-3-acetic acid (IAA) which belongs to the class of plant hormones called auxins. Today, plant hormones are defined as naturally occurring compounds, working at very low concentrations. In most cases they are transported throughout the plant body and interacting with specific receptor proteins and thus triggering the respective signalling pathway. Among the main group of hormones divided based on its chemical structure, belong: auxins, cytokinins, gibberellins, ethylene, abscisic acid, brassinosteroids, jasmonates and strigolactones (Frébort *et al*., 2011).

In the last years, important gaps in understanding of hormone metabolism and signalling have been filled. The major progress was achieved by identification of the key genes encoding enzymes that control critical steps of hormone biosynthesis, degradation, translocation or signal transduction (Werner & Schmülling, 2009). As these plant hormones can directly affect the plant growth and development, the genetic manipulation in the related genes has high potential to increase crop production or to develop stress-resistant plants. Unfortunately, there is no commercial transgenic plant with altered cytokinin metabolism, being released so far (Zalabák *et al*., 2013).

In the theoretical part of presented thesis, general introduction to cytokinin metabolism is discussed with the main part dedicated to the catabolic enzymes cytokinin dehydrogenases (CKXs). Experimental work is focused on transformation of plant material in order to reveal the subcellular localization of maize CKX isozymes. So far the information on cytokinin metabolism was obtained mainly from experiments on dicotyledonous model plant *Arabidopsis* (*Arabidopsis thaliana*). On the other hand, the information on this topic in monocotyledonous plants is limited. As the crop species are almost exclusively monocotyledonous, further information is necessary for deeper understanding of the CK metabolism and signalling for their growth and development.

Thus, maize (*Zea mays* L.) was selected for our experiments as a useful monocotyledonous model plant. Maize is one of the most important crop species used especially for direct human consumption, animal feed, and besides today being a significant plant source for chemical feedstock. Result of this work therefore partially contribute to elucidation of the subcellular distribution of cytokinin catabolism and thus expand the basic knowledge about cytokinin metabolism in monocots.

2 Current knowledge of the topic

2.1 Cytokinins

Cytokinins belong to important class of plant hormones and play a crucial role in various phases of plant development and growth, including shoot initiation and growth, apical dominance, cell division, leaf senescence, nutrient uptake and last but not least mediate the response to biotic and abiotic stress. Together with auxins function as long-distance messengers as well as local paracrine signals, while their synthesis and action take place at various sites in a plant body (Sakakibara, 2006). In order to be available in suitable concentration in the right time and at the right place and could therefore interact with the specific receptor, the hormone level must be precisely controlled. A key role in fine tuning of cytokinin level possess biosynthetic and metabolic enzymes as well as components of signal perception and transduction, whose major breakthrough was made only in the last decade.

Evolutionally, cytokinins are highly conserved low-mass molecules, which have been found in almost all known organisms. Their functions are not unique only to plants, but also to organisms such as bacteria, fungi, nematodes or e.g. insects (Spíchal, 2012). In 1950s, searching for compounds with cytokinin activity became intensive, when Miller and Skoog, discovered first cytokinin named kinetin as a DNA degradation product, promoting cell division, isolated from autoclaved DNA of herring sperm (Miller *et al*., 1955). Kinetin presence in plants was however confirmed more than 40 years later in dry coconut (Bar*cis*zewski *et al*., 1996).

2.1.1 Cytokinin function in plants

2.1.1.1 The cell cycle control

In mitotically active areas, such as the root and shoot meristem, high levels of cytokinin were revealed. Moreover, after exogenous application of cytokinin to some organs normally lacking this hormone has been shown to promote cell division and its connection to all stages of the cell cycle. Firstly, according to the experiments with cyclin CYCD3 over-expressing mutants, it was thought that cytokinins regulate cell cycle progression at the G1/S transition by inducing transcription of CYCD3 (Riou-KhamLichi *et al*., 1990). However recent studies on *ahk2/ahk3/ahk4* triple receptor mutant suggest that the key

point of cytokinin regulation in the cell cycle could be in the G2/M transition (Kieber & Schaller, 2014).

2.1.1.2 Root and shoot development

The shoot apical meristem (SAM) is a highly specialized group of pluripotent stem cells whose maintenance is essential for postembryonic formation of aerial organs and tissues. Consistent with results on cytokinin-deficient plants or knock-out lines of cytokinin biosynthetic genes or receptors, which led to smaller SAM, cytokinin was proved to be an essential regulator of proliferative SAM activity. Another confirmation of this view is localization of *LOG* genes expression in SAM, which encodes for enzymes converting biologically inactive cytokinin nucleotides to active free bases and so they serve as a source of active cytokinin. However, contribution of cytokinin as well as auxin signalling also modulates in SAM. Along with the antagonistic effect of auxin, cytokinin participates in the outgrowth of dormant axillary buds.

Cytokinins are known to inhibit root growth and development, since their discovery. The inhibitory role is associated with effects on cell division of the root meristem and on the cell expansion in root elongation zone. Size of root apical meristem (RAM) is determined by antagonistic action of cytokinins and auxins. High cytokinin/low auxin content regulates RAM size by promoting the rate of cell differentiation in the transition/elongation zone. On the other hand, high auxin along with low cytokinin concentration promotes cell proliferation and inhibits cell differentiation (Werner & Schmülling, 2009; Kieber & Schaller, 2014). In addition it was shown that cytokinin is a negative regulator of lateral root formation. The main mechanism of cytokinin action is through regulation of auxin flow, which is required for early stages in the development of lateral roost primordia. Correspond to this, cytokinin has the major effect on the expression and distribution of the PIN auxin efflux carriers in the root (Růžička *et al*., 2009; Zhang *et al*., 2011).

2.1.1.3 Leaf senescence

For years, cytokinins are known to inhibit leaf senescence. Studies on *Arabidopsis* cytokinin receptors or cytokinin responsive genes helped to elucidate mechanism of this process. According to the genetic analysis of three AHK receptors, AHK3 plays the key role, as its disruption displayed earlier leaf senescence, together with ARR2, type-B ARR of cytokinin responsive genes, which are most likely involved in regulation of this process

as well as cytokinin response factors (CRFs) (Kim *et al*., 2006). Disruption of *CFR6* gene results in decreased sensitivity of leaves to inhibitory effect of cytokinin. Surprisingly the overexpression of *CFR6* resulted in an even stronger acceleration of leaf senescence. Hence it is suggested that CRF6 might be involved in fine-tuning the timing of leaf senescence (Zwack *et al*., 2013).

2.1.1.4 Abiotic stress

Connection of cytokinins to various abiotic stresses has been reported. Cold stress rapidly induces expression of multiple type-A ARRs by AHK2/AHK3-dependent mechanism. Overexpression of type-A *ARR* genes enhanced freezing tolerance in *Arabidopsis* seedlings (Jeon *et al*., 2010). Cytokinin appears to play a role also in the response to drought and osmotic stress. Salt or osmotic stress has been showed to have a strong effect on the expression of AHK cytokinin receptors in *Arabidopsis*, leading to decreased level of cytokinins. Whereas AHK2 and AHK4 were down-regulated into root and shoot, AHK3 was up-regulated (Kieber & Schaller, 2014). Cytokinin involvement in drought stress was studied only recently, when expressed *IPT* gene from *Agrobacterium tumefaciens* under the control of stress-responsive promotor (SARK) in tobacco and cotton, resulting in high tolerance of plants to the drought stress. These findings suggest that elevated cytokinin level can enable plants to survive in drought conditions (Rivero *et al*., 2007; Kuppu *et al*., 2013). Interestingly, overexpression of *AtCKX3/AtCKX4* genes or multiple *IPT* disruption, which decreases endogenous cytokinin, resulted in both drought and salt-tolerant phenotype of *Arabidopsis* plants (Nishiyama *et al*., 2011).

Besides the main functions briefly described above, all other important roles of cytokinin and genes involved in these processes in plants are summarised in Fig. 1.

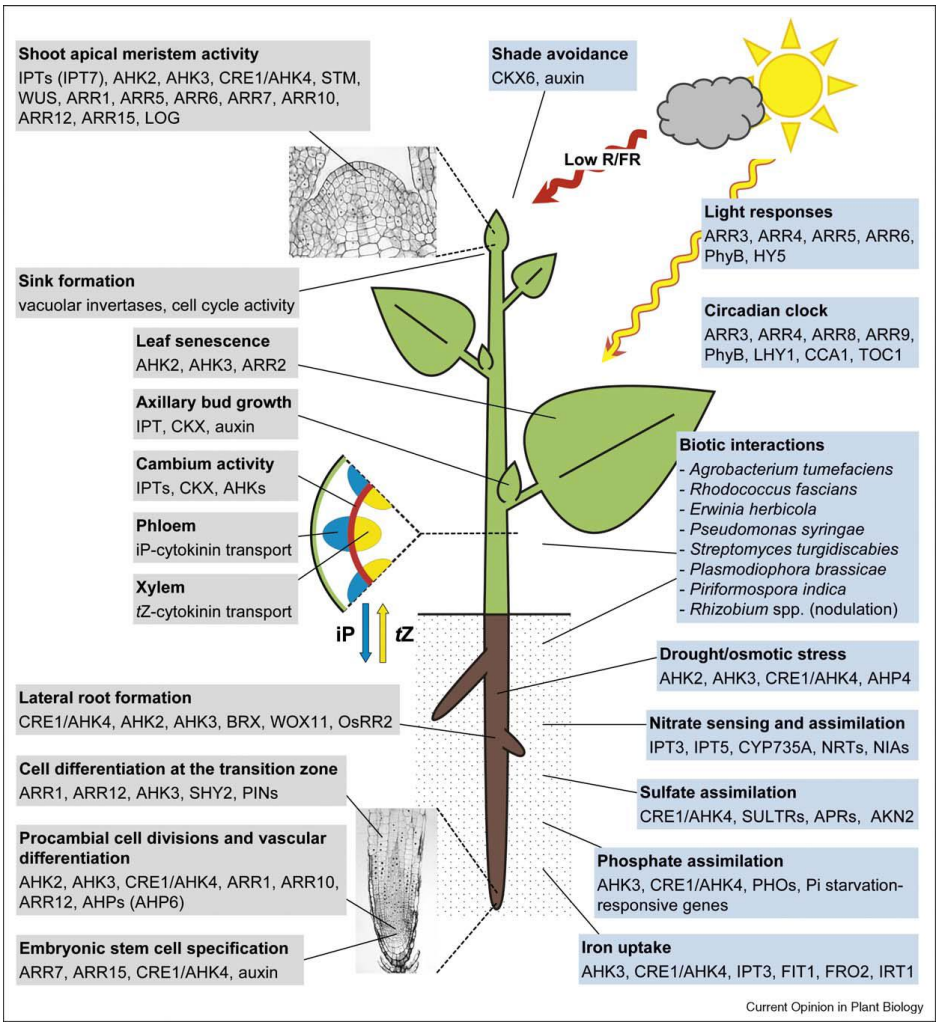

Figure 1: **Cytokinin actions in regulating plant development**. Adapted from Werner & Schmülling, 2009.

2.1.2 Occurrence and structure

Naturally occurring cytokinins are N^6-substituated derivatives of adenine, carrying either an isoprenoid or aromatic side chain (Fig. 2). The most prevalent forms are isoprenoid cytokinins, which can have either saturated or unsaturated aliphatic side chain. Isopentenyladenine (iP)- and *trans*-zeatin (*t*Z)-types of cytokinins (CKs) belong the major forms in *Arabidopsis*, whereas *cis*-zeatin (*c*Z)-type was reported to have low or no biological activity, but are present ubiquitously in the plant kingdom. Based on recent results, the possible function of *c*Z-type CKs as delicate regulators of CK responses in plants under growth-limiting conditions is hypothesized (Sakakibara *et al*., 2006; Gajdošová *et al*., 2011). Reduced form of zeatin – dihydroxyzeatin (DZ) occurs predominantly in storage organs like dormant seeds, apical buds or bean endosperm, and consistent with its resistance to degradation by CKX, it is suggested to serve as source of

active cytokinin immediately after germination (Galuszka *et al*., 2007; Frébort *et al*., 2011).

Unlike isoprenoid CKs, the second group is less frequent in plants and carries an aromatic side chain, which can be further substituted in different positions of an aromatic ring. Originally they were considered to be synthetic compounds with N^6-benzylaminopurine (BA), being the most active synthetic cytokinin. However BA and its hydroxyl- as well as methoxy-derivatives were actually found in several plant species. In poplar hydroxyl-derivatives of BA were identified: highly active *meta*-topolin and less active *ortho*-topolin, named after czech word for poplar-topol (Strnad, 1997; Tarkowská *et al*., 2003). Other compounds with cytokinin activity are synthetic derivatives of phenylurea, which exhibit the same activity in different CK bioassays and are effectively recognized by cytokinin receptors as adenine-type of cytokinins (Spíchal *et al*., 2004; Spíchal, 2012).

Cytokinin exist in plants in their active form as free bases, but also in the form of nucleosides/nucleotide or as conjugates with sugars at N^3, N^7 and N^9-position of the adenine ring, most commonly with glucose. N^9-position can be substituted with amino acid residues e.g. alanine or with methylthiol group at C^2-position. The primary ligands for cytokinin receptors are CK free bases, while sugar conjugates seem to be less or not active. (Spíchal *et al*, 2004; Sakakibara, 2006; Frébort *et al*., 2011).

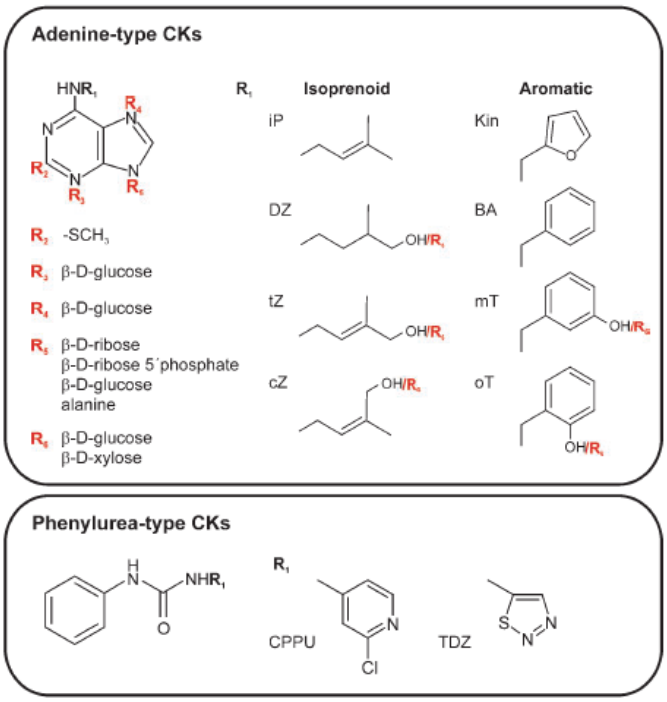

Figure 2: **Structure of cytokinins**. Adapted from Spíchal, 2012.

2.1.3 Transport and perception

At the beginning, it was thought that cytokinins are synthesized in roots and transported into shoot afterwards. However, further studies on expression of enzymes involved in cytokinin biosynthesis in *Arabidopsis* (mainly *IPT* and *LOG* gene families) indicated that synthesis of cytokinin takes place throughout the plant, including aerial tissues. It was proposed, that cytokinins serve primarily as long-distance messengers, but may act as paracrine signal as well, at least with respect to apical dominance and leaf senescence (Kieber & Schaller, 2014). In the xylem occurs shootward directed transport of cytokinins mainly in form of *t*Z-riboside, whereas in the phloem rootward transport of iP type cytokinins is mediated (Kudo *et al*., 2010). Beside the long distance transport, cytokinins must be transported across the plasma membrane into the lumen of endoplasmic reticulum (ER), as the primary site of cytokinin perception is there. Purine permeases AtPUP1 and AtPUP2, mediating CK nucleobase uptake, together with equilibrative nucleoside transporter AtENT6, proposed selective transporter of CK nucleosides, has been studied as possible candidates participating in membrane transport. Nevertheless there is no definitive demonstration so far (Sakakibara 2006, Kieber & Schaller, 2014) (Fig. 8).

The cytokinin signal transduction pathway is a phosphorelay (Fig. 3), using two component response system, which is similar to bacterial system responding to various environmental cues. Such a system generally consists of a membrane-localized sensor kinase, which recognizes environmental stimuli and a response regulator that propagates the signal, usually by regulating transcription of target genes. A family of cytokinin receptors described in *Arabidopsis* is composed of three histidine kinases AHK2, AHK3 and AHK4 (also called CRE1 or WOL1) are transmembrane proteins sharing a similar structure and following topology: extracellular portion called CHASE domain (cyclases/histidine kinases associated sensor extracellular) that functions in cytokinin binding, and cytosolic portion having histidine kinase and C-terminal receiver domains that comprise all the highly conserved residues required for enzymatic function. AHK receptors exhibit different affinities for various cytokinin species, e.g. AHK3 showed a high affinity for *t*Z and a relatively low affinity for iP. Contrasting with AHK2 and AHK4, which both exhibit high affinity for iP, so they can facilitate long distance communication between root and shoot as mentioned above. CRE1/AHK4, like some bacterial histidine kinases, has a dual function and works both as kinase and phosphatase in the presence or absence of cytokinin. In the absence of cytokinin, AHK4 acts as a phosphatase to

dephosphorylate AHPs, thereby decreasing signalling through the phosphorelay and serves as one of the possible negative regulators of cytokinin signalling (Mähönen *et al*., 2006a).

Downstream of the AHK receptors act *Arabidopsis* histidine-containing phosphotransfer proteins (AHPs). The AHP proteins function as mobile elements of the primary cytokinin signalling pathway, transferring the phosphate signal from the ER-localized cytokinin receptors to the nuclear-localized type-B ARRs. The *Arabidopsis* genome encodes five HPt proteins (AHP1 through 5) that contain the conserved residues required for activity, as well as one pseudo-HPt (APHP1/AHP6) that lacks the histidine phosphorylation site. *AHP6* expression is induced by cytokinin and functions as part of a negative feedback loop to reduce the sensitivity of a cell to cytokinin (Mähönen *et al*., 2006b).

Next components of cytokinin signalling pathway are A- and B-type of responsive regulators. Type-B ARRs are the key transcriptional factors that function in the final step of two-component signal system through cytokinin-regulated gene expression. Transcriptional activation has been proved by expression in yeast, showing direct binding to target DNA sequences through their GARP domain. Type-B ARRs have nuclear localization as expected (Lohrmann *et al*., 1999; Lohrmann, 2001). Genetic analysis based on loss-of function mutations identified only five out of the 11 type-B ARRs (ARR1, ARR2, ARR10, ARR11, and ARR12) as clearly functioning in the control of cytokinin signalling (Mason *et al*., 2005). *AtCKX4* has been identified as a cytokinin primary response gene that is directly regulated by the type-B ARR, ARR1, resulting in increased *CKX* expression, which causes concentration drop of bioactive cytokinins, thereby reducing signalling through the transduction pathway (Taniguchi *et al*., 2007).

On the contrary, the type-A ARRs act as negative regulators of cytokinin signalling. The *Arabidopsis* genome encodes 10 genes for type-A ARRs, which display high sequence similarity, but unlike the type-B ARRs, lack a classic output domain for transcriptional regulation (Zhang *et al*., 2001). Most of the type-A ARRs are transcriptionally induced in response to cytokinin and were, in fact, initially identified in screens for genes that are rapidly up-regulated by cytokinin in *Arabidopsis* (Taniguchi *et al*., 2007). The ability of the type-A ARRs to negatively regulate cytokinin signal transduction can be facilitate as follows: The type-A ARRs may compete with the type-

B ARRs for phosphorylation by the AHPs. Both type-A and type-B ARRs interact with the AHPs (Dortay *et al*., 2006). The transcriptional and post-transcriptional induction of type-A ARRs in response to cytokinin provides a strong negative feedback loop to supress the response to cytokinin, thereby limiting the output if the cytokinin activated phosphorelay in a cell (To *et al*., 2007).

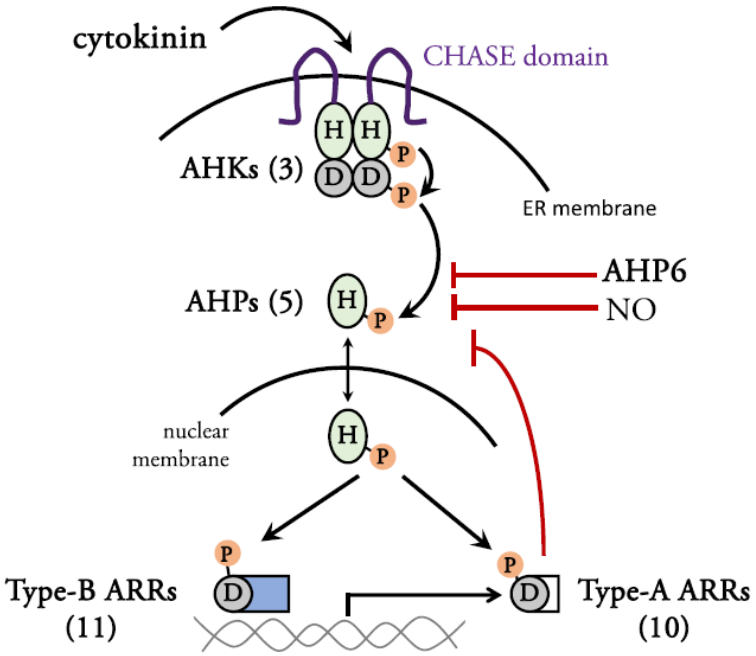

Figure 3: **Proposed model for phosphorelay signal transduction in cytokinin signalling**. Adapted from Kieber & Schaller, 2014.

2.2 Cytokinin metabolism

2.2.1 Biosynthesis

The first and rate-limiting step of cytokinin biosynthesis is an enzymatic reaction catalysed by isopentenyltransferases (IPTs), which mediate transfer of isoprenoid moiety to N^6-position of adenine either in its nucleotide (AMP, ADP or ATP) form or bound to tRNA. Two types of IPTs derived from the same ancestral gene are known to produce cytokinins in living organisms, differing in their substrates, namely: adenylate IPT (EC 2.5.1.27) and tRNA-IPT (EC 2.5.1.8). Donors of isoprenoid side chain can be either dimethylallyl pyrophospate (DMAPP) or (E)-4-hydroxy-3-methyl-but-2-enyl (HMBDP). Substrate specificities of IPTs vary depending on the origin and the species (Sakakibara, 2005; Spíchal, 2012).

2.2.1.1 *De novo* biosynthesis

First evidence of IPT-like activity was detected in extracts of the slime mould *Dictyostelium discoideum* (Taya *et al*., 1978), which is used for producing inhibitor of germination - discadenin, compound structurally related to cytokinins. The identification of *ipt* encoding gene was then identified in plant pathogenic bacteria *Agrobacterium tumefaciens* (Akiyoshi *et al*., 1984), namely *Tmr* gene (tumour morphology root), located on T-DNA region of Ti plasmid and as a substrates uses DMAPP and AMP. In *Arabidopsis thaliana*, *IPT* gene family was firstly described, containing nine homologs *AtIPT1-AtIPT9*. Except of *AtIPT2* and *AtIPT9*, which are suggested to encode putative tRNA-IPTs, AtIPT1 and AtIPT3-AtIPT8 were confirmed to be cytokinin synthases, more related to those in bacteria. However, unlike bacterial IPTs, AtIPTs use as substrates exclusively DMAPP and ATP or ADP, but not AMP (Takei *et al*., 2001; Kakimoto *et al*., 2001). For formation of *t*Z two routes were proposed. According to the first one, iP-dependent pathway, iP generates as first and after subsequent hydroxylation by cytochrome P450 monooxygenase enzymes, namely CYP735A1 and CYP735A2, *t*Z is produced. The second one, iP-independent pathway, assumes direct transfer of hydroxylated side chain, which was obtained from HMBDP via MEP pathway (Takei *et al*., 2004a).

There are two possible pathways of isoprenoid production in plants. Methylerythritol phosphate pathway (MEP), localized in plastids is the major source of prenyl group for *t*Z and iP, supported also by results of subcellular localization of AtIPT1, 3, and 8 in plastids. In cytosol/mitochondria, on the other hand, mevalonate pathway (MVA) provide prenyl group for *c*Z (Kasahara *et al*., 2004) (Fig.4).

2.2.1.2 tRNA degradation

tRNA-IPTs are present in almost all living organisms including bacteria, animals and plants. It was originally assumed that their breakdown could be the possible mechanism of cytokinins biosynthesis, however due to the slow turnover rate of tRNA it seemed to be rather insufficient for the amount of cytokinins produced in plants, with the possible exception of *c*Z. As for the structure, they share about 35% homology with adenylate IPTs, although they contain additional domain enabling binding to RNA. tRNA-IPTs catalyse modification of some mature tRNAs by *N*-prenylation of the adenine residue adjacent to the anticodon and proceeds by subsequent degradation into nucleotide

monophosphates. In most cases released cytokinin is *c*Z and because there is no evidence of *cis*-hydroxylase or zeatin isomerase since to date, tRNA is widely accepted as the exclusive source of *c*Z. Consistent to this, studies on deletion mutants of *AtIPT2* and *AtIPT9*, which are believed to function as tRNA-IPTs indicated decreased level of *c*Z type cytokinins, moreover being undetectable in their double mutant (Frébort *et al*, 2011; Spíchal, 2012).

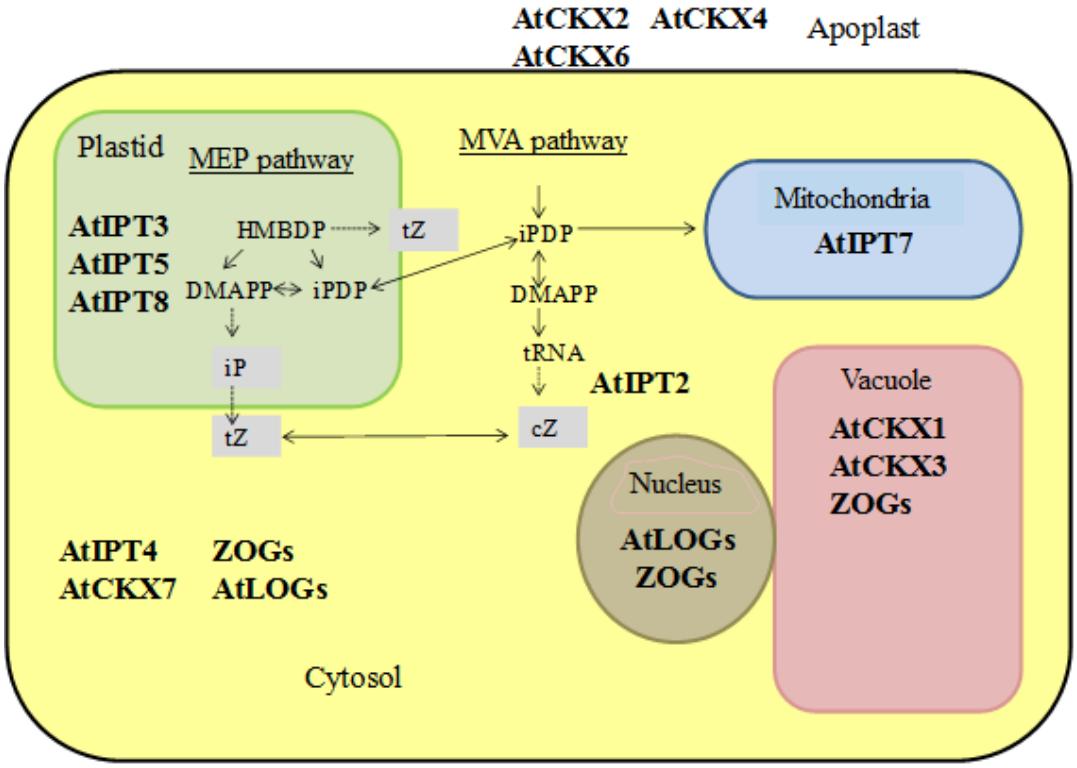

Figure 4: **Summary of cytokinin biosynthesis and distribution of metabolic enzymes in plant cell**. Adapted from Johnová, 2013, bachelor thesis.

2.2.1.3 Tissue and cellular expression of AtIPTs

Tissue expression pattern of individual IPTs in *Arabidopsis* examined using *GUS* fusion genes with regulatory sequence of each *AtIPT*, revealed that AtIPTs are distributed widely throughout the plant. AtIPT1::GUS fusion protein was found in xylem precursor cell files in the root tip, corresponding with possible involvement of cytokinin in xylem development, leaf axils, ovules and immature seeds. On the contrary, AtIPT3::GUS was expressed in the phloem almost throughout the plant. Similar expression pattern exhibit AtIPT4 and AtIPT8 in developing seeds, with highest expression in chalazal endosperm (CZE). Soon after germination, expression of AtIPT5::GUS in the columella root cap of

the primary root appeared, later also in root primordia, upper part of young inflorescence and fruit abs*cis*sion zones. AtIPT7::GUS was expressed in trichomes of young leaves, root elongation zone. Proposed tRNA-IPTs AtIPT2 and AtIPT9 were expressed ubiquitously with the stronger expression in proliferating tissues, including the root and shoot apical meristems and leaf primordia. It was shown that expression of AtIPTs undergoes hormonal control and responds also to nutrient status. *AtIPT6* is a pseudogene in *Arabidopsis* ecotype Wassilewskija (Ws), but a gene in ecotype Columbia (Col-0), however no expression signal was detected, suggesting the lack of necessary region downstream for its direct expression. Cytokinins downregulate the expression of AtIPT1, 3, 5 and 7, all commonly expressed in seedlings, indicating negative feedback regulation. Auxins up-regulate AtIPT5 and AtIPT7. Nitrates rapidly induce the expression of AtIPT3 in both roots and shoots, without requirement of cytokinin *de novo* biosynthesis. Expression of genes encoding for tRNA-IPTs were unaffected by mineral nutrients or phytohormones (Miyawaki *et al*., 2004). In other plant species similar effect of hormones was described and for example in maize, the levels of ZmIPTs were increased after stress treatment (Vyroubalová *et al*., 2009).

Subcellular distribution of AtIPTs was studied using *AtIPT* genes fused with GFP. Plastidial localization was proved in the case of AtIPT1, 3 and 5 as well as AtIPT8, however in diverse tissues, leaf or root cells, respectively. AtIPT4 and AtIPT2 were found in cytosol and AtIPT7 was targeted to mitochondria (Kasahara *et al*., 2004). Interestingly, localization of AtIPT3 is dependent on its farnesylation status, despite the presence of plastid-targeting signal peptide, farnesylation seems to direct most of the protein to the nucleus, while the non-farnesylated protein stays in plastids. It is assumed that there is a correlation between either iP-type or *t*Z-type cytokinin biosynthesis (Galichet *et al*., 2008) (Fig. 4).

2.2.2 Cytokinin interconversions

2.2.2.1 Lonely guy – cytokinin activation

De novo synthetized cytokinin nucleotides must be converted to their active form as free bases. Enzymes of general purine metabolism like various phosphatases such as 5-nucleosidase, deribosylases e.g. adenosine nucleosidases have a broad substrate specificity so for low-levels of cytokinin in plants, their affinity would be rather insufficient. Nevertheless, an enzyme catalysing reverse phosphorylation of nucleosides

to nucleotides adenosine kinase (ADK; EC 2.7.1.20), first studied in moss *Physcomitrella patens* (von Schwartzenberg *et al*., 1998) and displayed high affinity for CK ribosides in tobacco cells (Kwade *et al*., 2005). Recently, lines silenced in ADK expression were analysed in *Arabidopsis*, showing that despite high Km value for CK ribosides, significantly contributes to CK homeostasis (Schoor *et al*., 2011). Finally, the enzyme directly converting inactive CK ribosides into free-bases in one-step reaction was discovered in rice, by analysing *log* mutants deficient in the maintenance of shoot meristems, therefore named as LONELY GUY (LOG) (Kurakawa *et al*., 2007). The responsible gene, encodes a cytokinin riboside 5-monophosphate phosphoribohydrolase, which catalyses hydrolysis of CK 5-monophophate to CK nucleobase and ribose-5-monphosphate. Gene family of *LOGs* were confirmed in *Arabidopsis* and possess seven genes encoding active *AtLOG* (Kuroha *et al*., 2009). Disruption of multiple *LOG* genes lead to severe retardation of shoot and root growth and defects in maintenance of the apical meristems. It was shown that AtLOG4 and AtLOG7 play the major role in SAM, whereas AtLOG3 and AtLOG4 in the root (Tokunaga *et al*., 2012). Subcellular localization of AtLOGs revealed distribution in cytosol and nuclei, comparing with LOGs from rice, which tend to be localized only in cytosol (Kuroha *et al*., 2009) (Fig.4).

2.2.2.2 Glycosylation – cytokinin inactivation

Cytokinin level can be decreased through either conjugation to sugars or through irreversible cleavage by cytokinin dehydrogenases discussed below (Fig. 5). The most frequent modifications on adenine ring/isoprenoid chain are glycosylation mediated by UDP-glycosyltransferases (UGT, 2.4.1.X). These enzymes catalyse conjugation of sugar (glucose, xylose) from uridine diphosphate to cytokinin adenine ring or isoprenoid side to form *N*-glycosides or *O*-glycosides, respectively.

Adenine-ring conjugates

N-glycosylation occurs primarily on the N^7 or N^9 –positions of adenine, and due to the low or any activity in bioassays, it is supposed to be irreversible inactive forms of CKs. On the contrary, N^3-glycosides can be converted to the free bases by β-glucosidases (Brzobohatý *et al*., 1993). However, later studies showed that N^9-glucosides are preferred substrates for some CKX isoforms (Galuszka *et al*., 2007; Kowalska *et al*., 2010).

Isoprenoid side chain conjugates

Cytokinins containing hydroxyl group on the isoprenoid side chain (zeatin-type of CKs; topolins) undergo *O*-glycosylation. *O*-glycosylation is stereo-specific reaction with strict preferences for CK substrates. For example *O*-glucosyltransferase from *Phaseolus lutenus* ZOG1 recognizes only tZ isomer and uridine diphosphate glucose (UDPG) or uridine diphosphate xylose (UDPX) as sugar donor, while *cis*ZOG1 and *cis*ZOG2 isolated from maize cannot recognize *t*Z or DZ, but only *c*Z can serve as a substrate and use UDPG as donor of sugar (Veach *et al*., 2003). Unlike N^7- and N^9-glycosides, *O*-glycosides (together with N^3-glycosides) can be effectively deglycosylated by β-glucosidase, thus because the ability of *O*-glycosides to convert back to the active CKs, they are considered as important storage forms of cytokinins. Surprisingly, *O*-glycosyltransferases from *Arabidopsis* do not make a difference between two zeatin isomers, on the contrary they utilize both with the same effectivity (Hou *et al*., 2004).

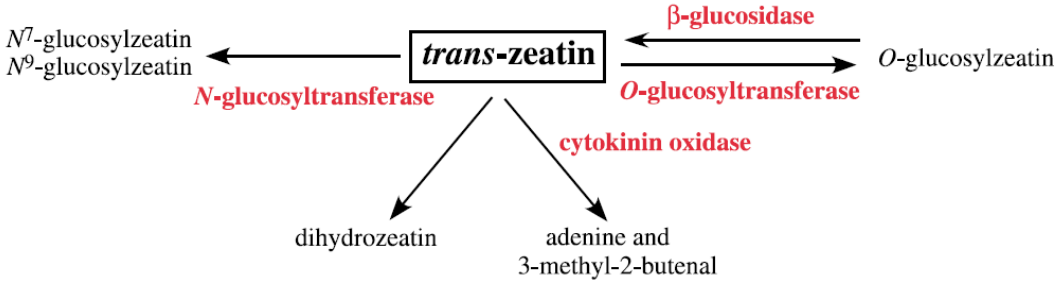

Figure 5: **Metabolic fates of *trans*-zeatin**. Adapted from Kieber & Schaller, 2014.

2.2.3 Irreversible degradation

Cytokinin dehydrogenase (CKX; EC 1.5.99.12) is the only known enzyme responsible for irreversible degradation of cytokinins mediated by oxidative cleavage of the side chain from adenine ring. CKXs significantly contribute to maintaining the endogenous cytokinin levels and metabolic regulation of cytokinin-dependent processes. CKX activity was first demonstrated in crude extracts from tobacco tissue by conversion of radiolabelled N^6-isopentenyladenin to adenine (Pačes *et al*., 1971). Similar activity was later found in maize kernels. The enzyme has been named cytokinin oxidase (CKO) and incorrectly classified as copper amine oxidase (Whitty and Hall, 1974). Despite it was

originally assumed that the enzyme utilises exclusively molecular oxygen as an final electron acceptor, so function as oxidase, further studies showed variety of electron acceptors other than oxygen that can be used, moreover they are strongly preferred to oxygen. Therefore the enzyme was reclassified as cytokinin dehydrogenase (Galuszka *et al*., 2001).

2.2.3.1 Structure and mechanism of reaction

CKXs belong to the class of oxidoreductases with covalently bound flavin adenine dinucleotide (FAD) serving as a cofactor. Molecular weight of CKX proteins ranges between 56 - 64.9 kDa, differences are often caused by posttranslated-formed glycosylation (Schmulling at al., 2003). The crystal structure of two mature CKX, namely ZmCKX1 (Fig. 6) and AtCKX7 was identified so far (Malito *et al*., 2004; Bae *et al*., 2008) and revealed highly conserved active site in terms of amino acid composition as well as in architecture. CKXs contain two domains, for FAD and for substrate binding, respectively. Covalent binding of FAD to protein is facilitated through 8-methyl group of the isoalloxazine ring to histidine residue of well-conserved GHS motif situated in the N-terminal part of the enzyme. The catalytic centre consists of a tunnel-shaped region on the protein surface and an internal cavity lined by the flavin ring. Substrate binding above the isoalloxazine plane does not cause any conformational changes (Malito *et al*., 2004).

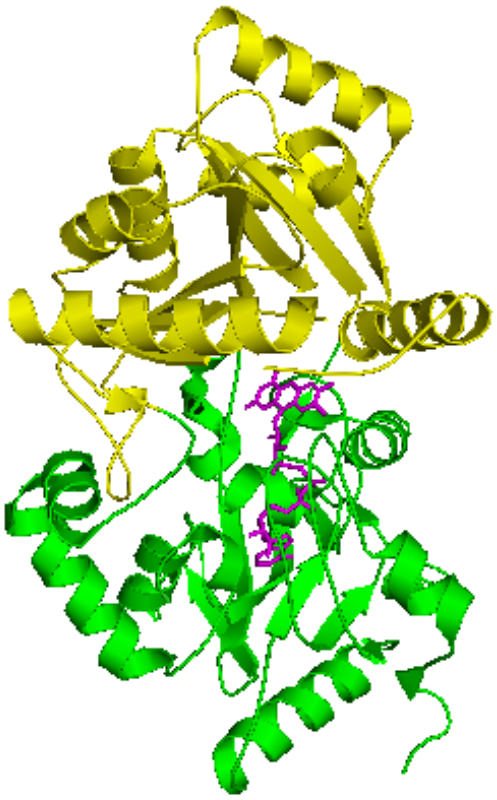

Figure 6: **Crystal structure of ZmCKX1 monomer**. Yellow region binds cytokinin and green region binds FAD cofactor, which is marked in purple. Modified according to Malito *et al*, 2004.

The reaction, which catalyses the breakdown of cytokinin is achieved by oxidative cleavage of N^6-side chain of adenine (Fig. 7). First step is dehydrogenation, when two-electron are transferred from the CK substrate to an FAD cofactor, followed by the formation of an imine intermediate and simultaneous removing of two electrons from FAD to an electron acceptor. The reaction proceeds via hydrolysis of the intermediate, so the final products are adenine or adenine derivatives (for N^9-substituated cytokinins) and corresponding unsaturated aldehydes derived from the side chain. As mentioned earlier, CKXs effectively use a variety of artificial electron acceptors under anaerobic conditions. Biochemical studies on the ZmCKX1 reaction mechanism suggested preference of quinones, generated from plant phenolics by peroxidase. Recently, it was shown that as the most efficient natural occurring electron acceptors of CKX can serve products of benzoxazinone oxidative degradation and their free radicals. Such compound 2,4-dihydroxy-7-methoxy-1, 4-benzoazin-3-one (DIMBOA) was found to be abundant secondary metabolite in maize and was examined in this context (Frébortová *et al*., 2010).

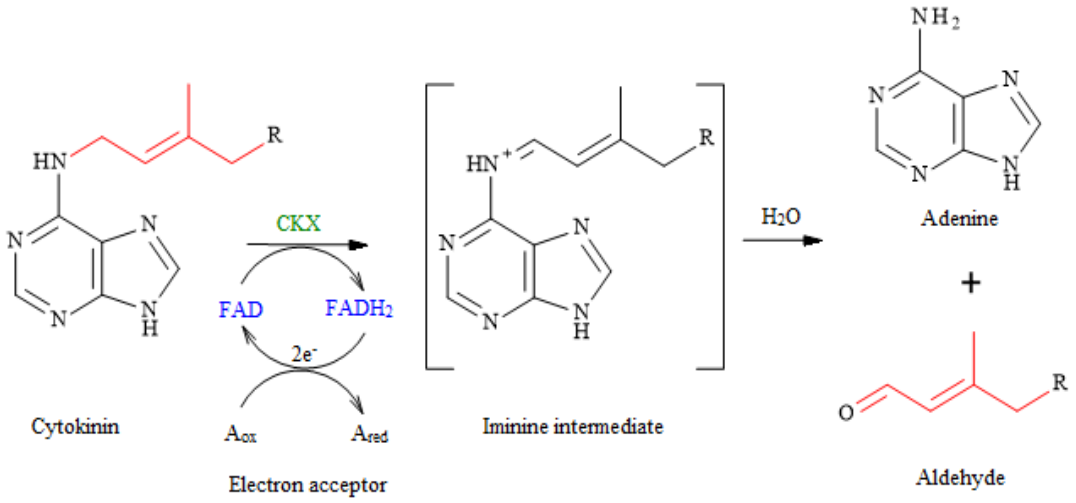

Figure 7: **Reaction scheme of cytokinin dehydrogenase reaction**.

2.3 Cytokinin dehydrogenase family

2.3.1 Introduction

Cytokinin dehydrogenases have been described in wide range of plant species, usually encoding small gene families with varying number of members. Biochemical properties, patterns of tissue expression or intracellular localization differ within each family, but also in between monocot and dicot plants (Werner *et al*., 2006). The major breakthrough was achieved by identification and cloning of the first *CKX* gene, *ZmCKX1* isolated from maize kernels (Houba-Hérin *et al*., 1999; Morris *et al*., 1999).

CKXs exhibit relatively high sequence homology and contain several conserved domains. These include the GHS domain, which is responsible for the covalent binding of FAD cofactor. Other unique protein motifs typical for CKX are the PHPWLN and HFG located in the C-terminal region. Both motifs are strongly conserved among monocot CKXs and those in *Arabidopsis*, however HFG is supposed to be crucial for activity and stability of CKX enzymes (Zalabák *et al*., 2014). Individual CKX isoforms show high divergence in the N-terminal sequences, which determine the targeting of mature CKX proteins to various cell compartments. In general, there is usually only one isoform localized in cytosol, thus lacking N-signal peptide, and all the others are vacuolar or associated with ER, later most probably directed to the apoplast (Werner *et al*., 2003).

Distinct subcellular localization probably influence enzyme function and is essential for the physiological consequences of its disturbed homeostasis after genetic manipulation. Moreover, localization of CKXs in the cell is most likely closely related to preferences for different substrates (Zalabák *et al*., 2013).

2.3.2 *Arabidopsis thaliana* cytokinin dehydrogenases

CKX gene family in *Arabidopsis* is the most intensively studied and completely analysed CKX family from all the plant species. It contains seven homologous genes *AtCKX1 to AtCKX7* (Fig. 10). Tissue expression pattern of respective *AtCKX* genes was studied using GUS expression driven by native promotor regions. It was confirmed that AtCKX1 is expressed in the vascular cylinder of lateral roots, while AtCKX2 showed strongest expression in the shoot apex and AtCKX3 in young shoot tissues. In regions, mostly with high mitotic activity – developing trichomes, stomata, stipules and the root cap AtCKX4 was expressed. Strong expression in stamen, primordial and developing pollen was reported by AtCKX5, whereas AtCKX6 was expressed in the gynoecium at various stages of development (Werner *et al*., 2003).

According to the presence of N-terminal sequences in AtCKXs proteins, prediction programs (TargetP, PSORT) indicate that the four of them – AtCKX2, AtCKX4, AtCKX5 and AtCKX6 are targeted to the plant secretory pathway, whereas AtCKX7 seems to be localized in cytosol, due to the lack of any signal peptide. AtCKX1 and AtCKX3 were initially predicted to be targeted to mitochondria. Subcellular localization of AtCKX-GFP fusion proteins revealed vacuolar targeting of AtCKX1 and AtCKX3, the rest with the exception of cytosolic AtCKX7, is secreted to the apoplast (AtCKX2-AtCKX6) (Werner *et al*., 2003). However, expression of AtCKX1 and AtCKX3 in epidermal leaf cells indicate localization in ER, whereas in root cells in vacuoles. Therefore it is suggested that vacuolar targeting is specific only for some cell types, where transfer from ER lumen to vacuoles takes place (Fig. 8).

As for the substrate specificity of AtCKXs isoforms, it was shown, that secreted isosymes prefer CKs free bases, whereas vacuolar and cytosolic utilize rather N^9-glycosylated CKs. However, later was shown that vacuolar CKXs have higher preference for CK di- and tri- phosphates, primary products of *de novo* CK biosynthesis and suggesting that spatial separation of IPT and CKX activity must be properly maintained (Galuszka *et al*., 2007; Kowalska *et al*., 2010). This also corresponds with the presumed

occurrence of particular cytokinin derivatives in different cell compartments, which illustrates active control of cytokinin levels throughout the cell as well as throughout the plant organs.

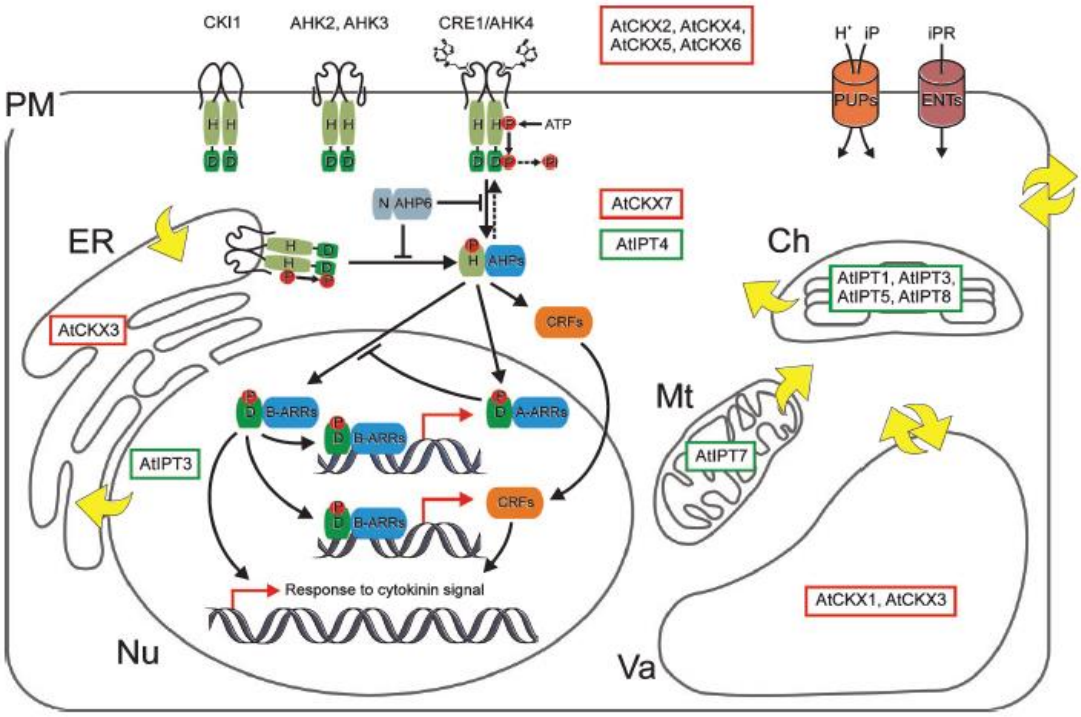

Figure 8: Model of CK signalling and subcellular distribution of biosynthetic and catabolic enzymes in *Arabidopsis thaliana*. Adapted from Spíchal, 2012.

The constitutive expression of *AtCKXs* in *Arabidopsis* and tobacco resulted in cytokinin-deficient plants, having prominent alterations in plant phenotype and revealing distinct role of CKXs in root/shoot growth, due to almost half-decrease of CK content. Strong morphological abnormalities such as stunned shoots with smaller apical meristem, prolonged plastochrone, slower leaf cell production and excessive root system development were observed (Fig. 9). Interestingly, overexpression of vacuolar localized AtCKX isoforms caused more severe CK deficiency syndrome compared with the apoplastic ones (Werner *et al.*, 2001; Werner *et al.*, 2003). Inactivation of single *CKX* gene does not lead to such strong phenotypic change like in double *atckx3/atckx5* knock-out line, showing that these isoforms play a key role in formation of inflorescence meristems (Bartrina *et al.*, 2011). Recently, a detailed study of cytosolic *AtCKX7* overexpressing lines revealed different phenotype patterns contrasting with plants overexpressing other *AtCKX* isoforms. Transgenic plants expressing *CaMV35S::CKX7-*

GFP developed short, early terminating primary roots with small apical meristem and vascular bundles of primary root contained only protoxylem elements, thus resembling the *wol* mutant of the *CRE1/AHK4* receptor gene, which was showed to be required for the AtCKX7 phenotype. Depletion of several cytokinins, mainly *c*Z-type and N-glucosides was observed, when compared to other CKX overexpressing plants. Therefore, it was hypothesized that the pool of cytosolic cytokinins is particularly relevant in the root procambium where it mediates the differentiation of vascular tissues through CRE1/AHK4 (Köllmer *et al*., 2014).

Tissue-specific expression as well as subcellular compartmentalization are processes involved in regulation of endogenous cytokinin content, is important for ensuring the proper regulation of cytokinin function in plants and each gene is more or less involved in specific developmental and physiological functions.

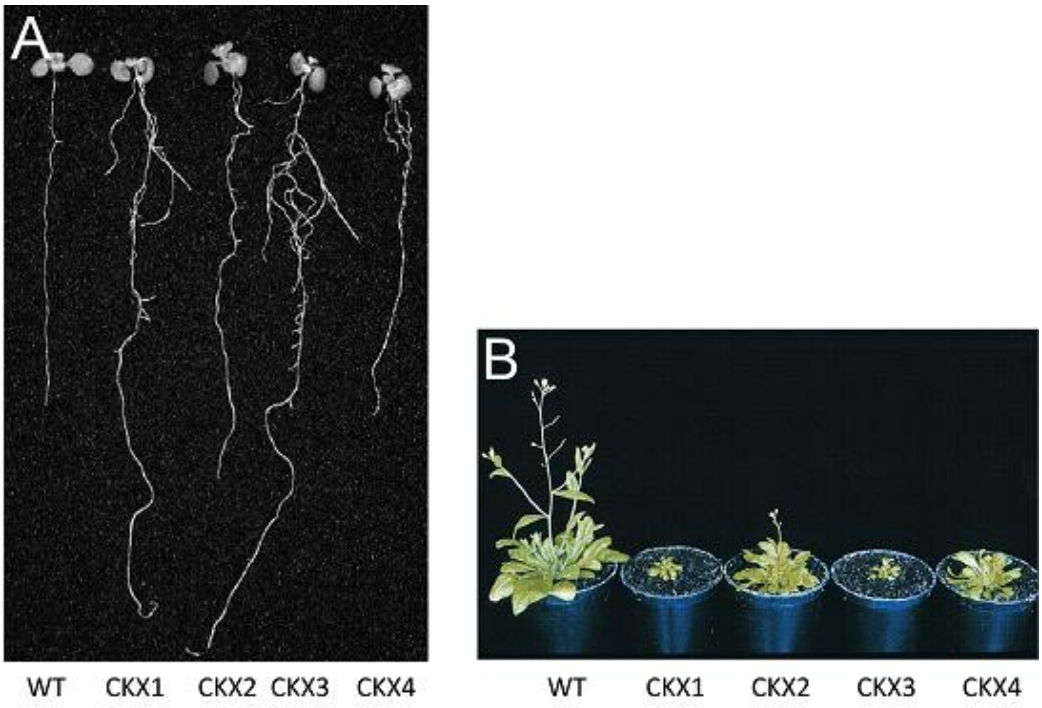

Figure 9: **Phenotypes of *Arabidopsis thaliana* plants overexpressing *AtCKX* genes under the control of *CaMV35S* promoter**. Adapted from Werner *et al*., 2003.

2.3.3 Maize cytokinin dehydrogenases

The maize genome encompasses thirteen genes encoding for cytokinin dehydrogenases ZmCKX1 to ZmCKX12, as the monocot species tend to have more members. The entire maize CKX gene family was characterized in detail only recently, not much has been known before. There are several closely homologous CKX gene pairs in the maize genome (Fig. 10) that were formed during recent chromosome duplication events. Gene pairs forming very close paralogs are *ZmCKX2/ZmCKX3*, *ZmCKX4a/ZmCKX4b*, *ZmCKX7/ZmCK8* and ZmCKX11/ZmCKX12, respectively (Vyroubalová *et al*., 2009).

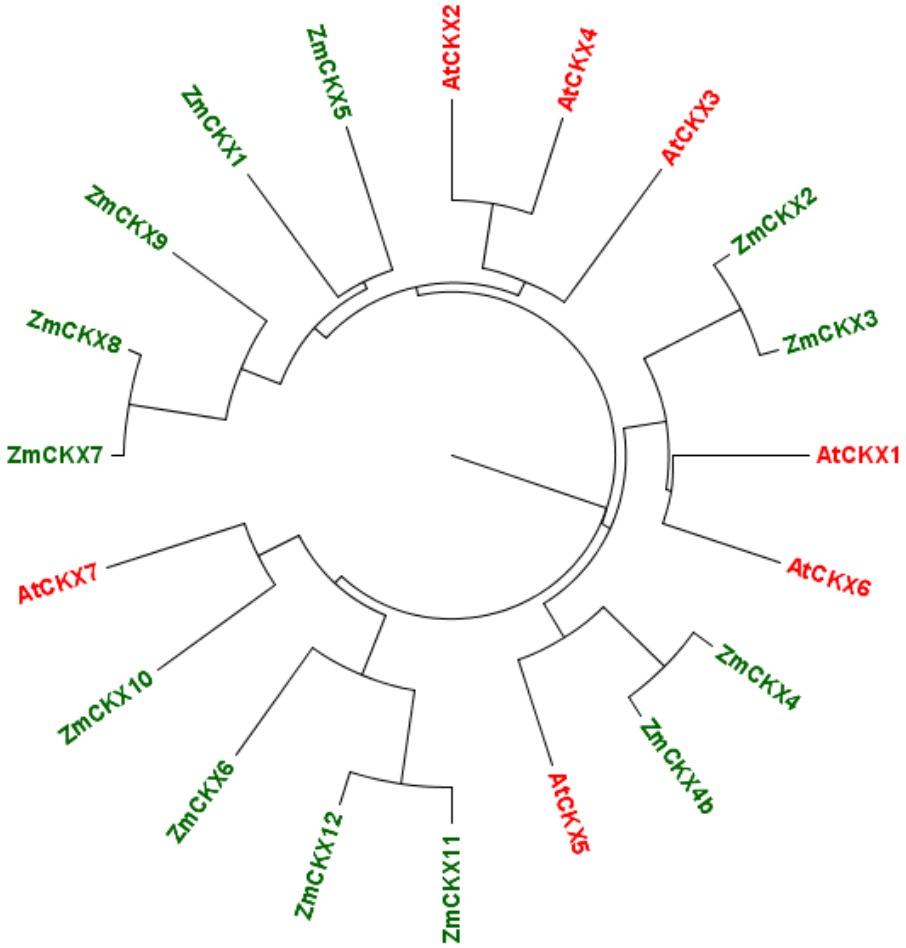

Figure 10: **Unrooted tree of cytokinin dehydrogenases from *Arabidopsis thaliana* and *Zea mays***. Protein sequences were processed using ClustalX2 program and the tree was visualized in FigTree 1.4.2.

Expression pattern of the whole maize *CKX* gene family was analysed in 7-d-old roots and leaves as well as in various tissues of mature plants along with developing and germinating kernels. The transcript levels of *CKX* genes don't seem to be higher than those of *IPT*s. The only cytosolic CK degradation enzyme, ZmCKX10, is relatively strongly expressed in all maize tissues with the exception of endosperm. Other CKXs probably have a redundant role or can be expressed in specialized cell types in different plant tissues; hence, it is clear from their low abundance in all materials tested (e.g. ZmCKX7 or ZmCKX9). There are some paralogs predominantly bound to the reproductive organs (i.e. ZmCKX4a and ZmCKX5). It was shown that ZmCKX1, ZmCKX6, and ZmCKX8 expressed in young roots and ZmCKX3, ZmCKX6, and ZmCKX11 expressed in young leaves most likely contribute to the pool of CK degradation activity in tissues exposed to the stress conditions (Vyroubalová *et al*., 2009)

As the subcellular localization of each CKX isoform is determined by an N-terminal sequence motif, predictive tools (SignalP 4.0, iPSORT) for identifying potential N-terminal signal peptides were used. In all studied ZmCKX sequences, namely ZmCKX1, 2, 3, 4a, 4b, 5, 6, 8, 9 and 12, putative N-terminal signal peptides were found, with the only exception of ZmCKX10, which was previously found to be localized in the cytosol due to the missing N-terminal signal sequence (Šmehilová *et al*., 2009). According to the prediction programs, ZmCKX1-6, ZmCKX9, ZmCKX11 and ZmCKX12 isoforms seem to be secreted to the apoplast, while ZmCKX8 was predicted to be transported to the mitochondria (Zalabák *et al*., 2014). Similarly, two isoforms of AtCKX were firstly predicted to have a mitochondrial signal, however later was shown that they actually are localized in the vacuoles (Werner *et al*., 2003). Subcellular localization of ZmCKX1was first studied using imunohistochemical methods and confirmed secretion to the apoplast. Highest expression as well as enzymatic activity was measured in the aleurone layer of maize kernels (Galuszka *et al*., 2005). In another study, C-terminal fusions of ZmCKX1 and ZmCKX10 with GFP were used for determination of subcellular localization. Heterologous expression of ZmCKX-GFP proteins in tomato hairy roots confirmed previously observed apoplastic localization of ZmCKX1, whereas ZmCKX10 targeting to cytosol and nucleus, which correspond to the lacking signal peptide at N-terminus. Any significant changes in phenotype of transgenic plants overexpressing ZmCKX1 and ZmCKX10 were observed. The only difference was in the amount and speed of tissue production. While roots expressing ZmCKX1 formed faster comparing with wild-type,

retarded growth of roots expressing ZmCKX10 was observed, resulting in lower tissue production (Šmehilová *et al*., 2009; Fig. 11). Overexpression of cytosolic isoform seems to have distinct impact on plant phenotype, which is in agreement with recent studies on *AtCKX7* overexpressors (Köllmer *et al*., 2014).

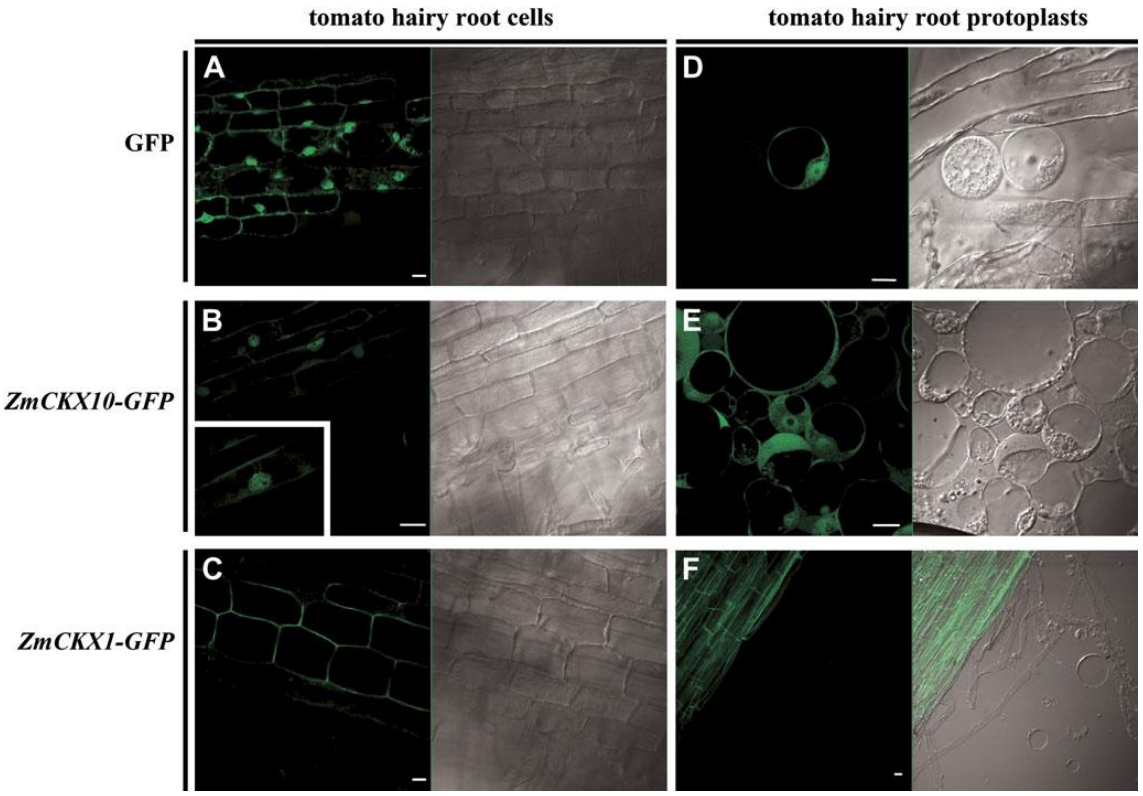

Figure 11: **Subcellular localization of ZmCKX1 and ZmCKX10 in tomato hairy roots**. Adapted from Šmehilová *et al*., 2009.

Recently ten recombinant ZmCKX isozymes were prepared using heterologous bacterial expression in *E. coli*. In order to express individual enzymes, it was necessary to remove putative N-terminal sequences. Detectable CKX activity was measured in cell lysates of only eight isoforms except ZmCKX6 and ZmCKX9. Further analysis of ZmCKX6 and ZmCKX9 sequence revealed presence of short sequence, downstream of the predicted signal peptide. This sequence was homologous to a sequence of LLPT and LPTS vacuolar sorting signals, respectively. When this motif was removed from N-terminus of ZmCKX9, functional enzyme was expressed. On the contrary, no CKX activity was recorded in the case of ZmCKX6 (Zalabák *et al*., 2014). It was assumed that coding sequence of ZmCKX6 is a pseudogene, however strong expression in intact maize tissues were formerly detected (Vyroubalová *et al*., 2009). ZmCKX6 has an unusual variation in the conserved PHPWLN motif, where first proline is changed to histidine

(HHPWLN), and also mutation in HFG motif, carrying leucine instead of phenylalanine, HLG. The reverse mutations in conserved HFG motif resulted in increased level of ZmCKX6 expression, but not in the case of reverse mutation of PHPWLN. This experiment clearly proves that HFG motif is necessary for the correct function of the CKX enzymes (Zalabák *et al*., 2014).

In contrast to most of the AtCKX family members, maize CKXs are generally able to degrade *c*Z-type cytokinins, often at rates higher than those for *t*Z-type CKs. It is probably due to the high abundance of *c*Z-type cytokinins in maize tissues, while in *Arabidopsis* they are abundant only in seeds (Gajdošová *et al*., 2011). In *Arabidopsis* vacuolar and cytosolic CKXs strongly prefer cytokinin-9-glucosides and cytokinin mono-, di- and tri-phosphates over the CK free bases (Kowalska *et al*., 2010). Conversely, maize CKXs degrade cytokinin monophosphates and zeatin-9-glucosides more slowly than free bases. The main isoform responsible for the rapid degradation of cytokinin free bases in the extracellular space is ZmCKX1. None of the tested ZmCKXs was able to utilize DZ as a substrate (Zalabák *et al*., 2014).

Experimental part

3.1 Material

3.1.1 Chemicals

2-(1-naphthyl)acetic acid (NAA) (Sigma, USA), 2.4-dichlorophenoxyethanoic acid (2,4-D), Acetic acid (CH3COOH) (Lachema, Czech Republic), Acetosyringon (Sigma-Aldrich, USA), Acrylamide (Sigma-Aldrich, USA), Agar (Merck, Germanny and HiMedia, Indie), Agarose (Amresco, USA), Albumin, bovine serum (BSA) (Sigma-Aldrich, USA), Amide black 10 B (Merck, Germany), Ammonium persulphate (APS) (Sigma-Aldrich, USA), Ampicilin (Sigma-Aldrich, USA), Bromphenol blue (Fluka – Sigma-Aldrich, USA), Coomassie Brilliant Blue G250 (Serva, USA), Dimethylsulfoxide (DMSO) (Duchefa, Netherlands), Ethanol (Penta, Czech Republic), Ethidium bromide (NeoLab, Germany), Ethylendiamintetraacetic acid (EDTA) (Penta, Czech Republic), Glycerol (Lach-Ner, Czech Republic), Hydrochloric acid (HCl) (Penta, Czech Republic), Hygromycin B (Roche Diagnostics, Switzerland), Isopentenyl adenine (OlChemIm Ltd., Czech Republic) Kanamycin monosulphate (Duchefa, Netherlands), Kinetin (Sigma-Aldrich, USA), Milk powder Laktino (PML Protein.MLéko.Laktóza, a.s., Czech Republic), Murashige and Skoog including vitamins (Duchefa, Netherlands), N, N´-methylenbisacrylamide (bisacrylamide) (Sigma-Aldrich, USA), Phenylmethansulfonylfluoried (PMSF) (Sigma-Aldrich, USA), Phytagel (Sigma-Aldrich, USA), Rifampicin (Duchefa, Netherlands), Sodium dodecyl sulphate (SDS) (Penta, Czech Republic), Sodium hydroxide (NaOH) (Penta, Czech Republic), Sodium chloride (NaCl) (Lach-Ner, Czech Republic), Sucrose (Penta, Czech Republic), Tetramethylendiamin (TEMED) (Sigma-Aldrich, USA), Timentin (Duchefa, Netherlands), Trihydrophosphoric acid (Lach-Ner, Czech Republic), Tris (Duchefa, Netherlands), Triton X100 (Pancreac, Spain), Trypton (Duchefa, Netherlands), Tween 20 (NeoLab, Germany), Yeast extract (Sigma-Aldrich, USA), β-merkaptoethanol (Sigma-Aldrich, USA).

Providers of all other chemicals including enzymes, purification kits etc. are listed below, in each method. Final concentrations of antibiotics are listed in brackets.

3.1.2 Bacterial strains and cultivation

- *Escherichia coli*, TOP 10 Electrocompetent cells (Invitrogen, USA)
- *Agrobacterium tumefaciens* GV3101, electrocompetent cells (Invitrogen, USA)

Both bacteria strains where cultivated in LB medium (Tab. 1) in appropriate volume on the rotary shaker. *Escherichia coli* (*E. coli*) was grown at 37 °C and *Agrobacterium tumefaciens* (*A. tumefaciens*) at 28 °C. According to the plasmid vector (Tab. 2), respective antibiotic selection was used.

Tab.1: Composition of LB medium.

LB medium
10g/L Tryptone
10g/L NaCl
5g/L Yeast Extract
adjust pH 7.2 (NaOH), autoclave

3.1.3 Plasmid vectors

Tab. 2: Plasmid vectors used for cloning and other.

	Promotor	**Antibiotic resistance*** Bacteria/Plants	**Source**
pBRACT-GFP	Ubi1	Kan/-	-
pGWB5	CaMV35S	Kan; Hyg B/HygB	Nakagawa *et al*. (2007)
pLNU-GFP	Ubi1	Amp/-	DNA c.s.
pSEG36-EGFP	CaMV35S	Amp/Kan	Prepared in this work
ER-TdTomato construct	CaMV35S		Dirck Becker, University of Hamburg
pENTR2B	T7	Kan, CmR/-	Invitrogen, USA

*Amp – ampicillin (100 mg/L)
Hyg B- hygromycin B (50 mg/L)
Kan – kanamycin (50 mg/L)

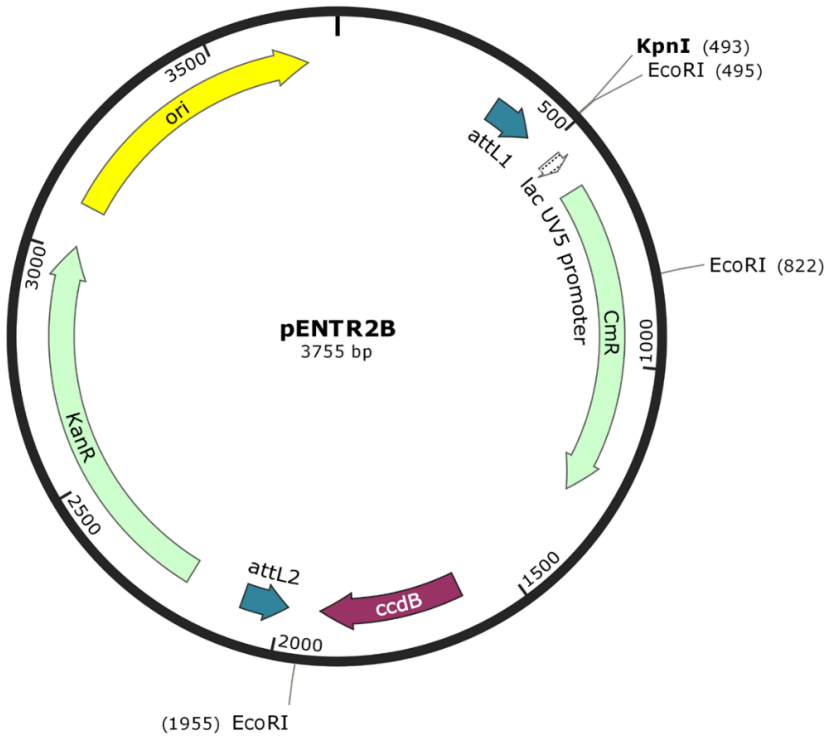

Figure 12: **Map of entry vector pENTR2B**. Ori – origin of replication (yellow), KanR - gene for kanamycin resistence (light green), CmR – confers resistance to chloramphenicol (light green), recombination attL sites (blue) and ccdB – gene encoding inhibitor of DNA gyrase (purple). Restriction sites of EcoRI and KpnI used for cloning of *ZmCKX* genes are displayed.

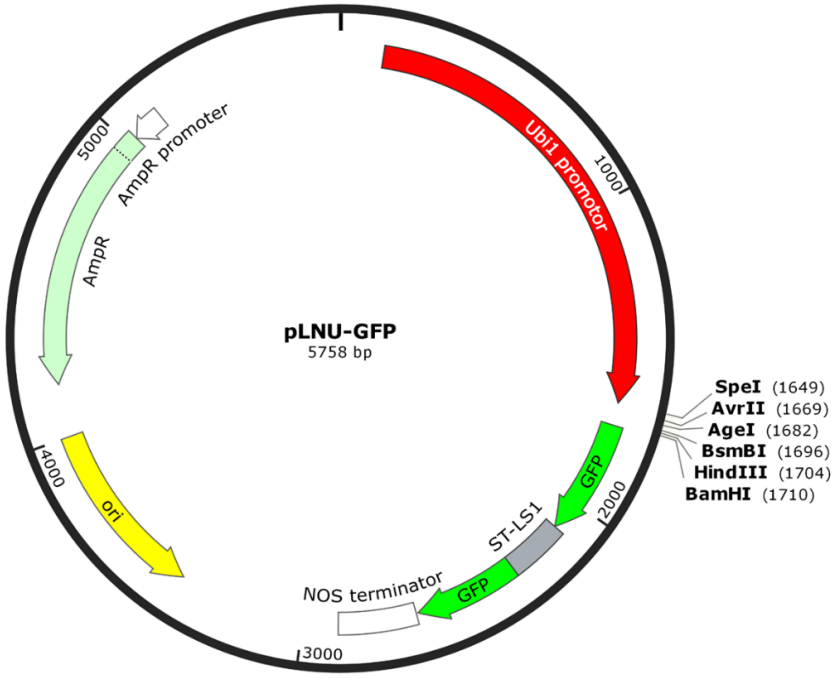

Figure 13: **Map of expression vector pLNU-GFP**. Ubi1 promotor - ubiquitin 1 maize promotor (red), GFP – gene for green fluorescent protein (green), ST-LS1 – intron (gray), NOS - nopaline synthase terminator (white), Ori – origin of replication (yellow), AmpR – gene for ampicillin resistance (light green). Multi cloning site represented by individual restristriction sites.

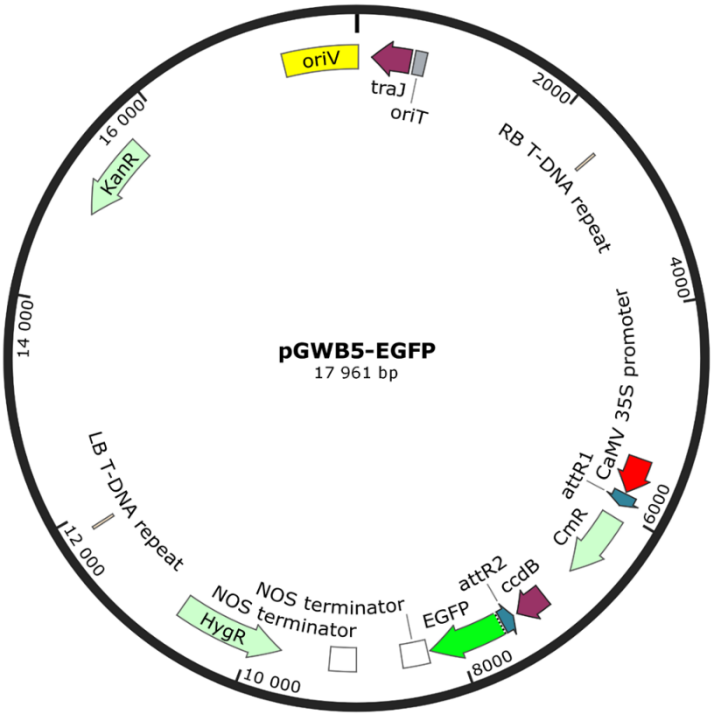

Figure 14: **Map of expression vector pGWB5-EGFP**. CaM35S – constitutive promoter from cauliflower mosaic virus (red), attR recombination sites for Gateway cloning (blue), ccdB - gene encoding for DNA gyrase inhibitor (purple), EGFP – enhanced GFP, NOS – nopaline synthase terminator (white), HygR – confers resistance to hygromycin, LB and RB T-DNA – left and right border repeats from nopaline C58 T-DNA. , KanR – confers resistance to kanamycin OriV – origin of replication (yellow).

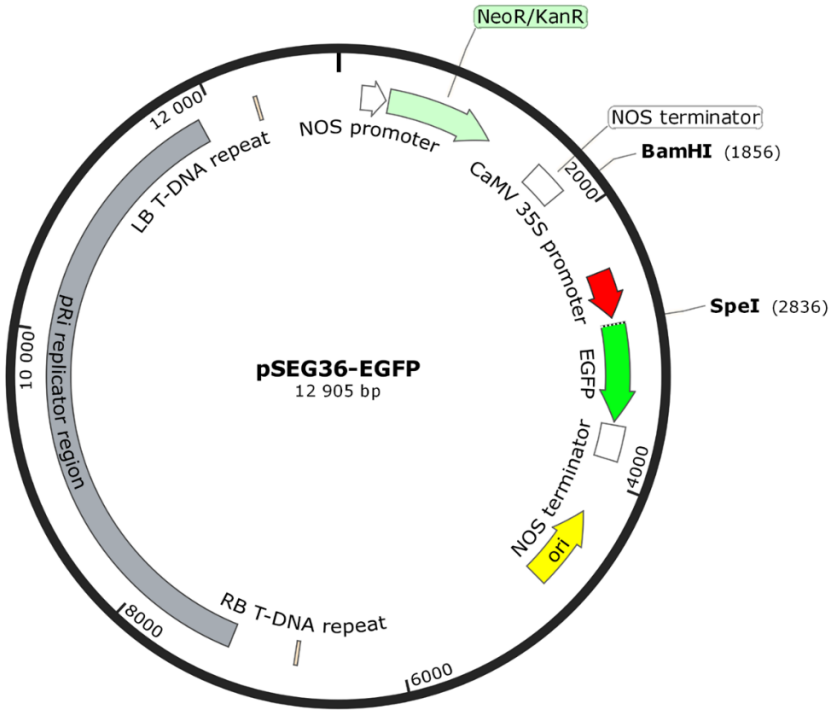

Figure 15: **Map of pSEG36-EGFP vector**. pRi - replicator region from *Agrobacterium* plasmid (gray), restriction sites used for cloning. All other features are described in the figure above.

3.1.4 Primers

Primers were supplied by Metabion (Germany).

Tab. 3: List of primers used for cloning into pENTR2B dual selection vector.

ZmCKX4ab KpnI + Kozak fw
5'-CGG GGT ACC CCA TGA CGC GGT GCC TCA TGT TC-3'
ZmCKX4aR wo STOP EcoRI
5'-CGG AAT TCC GCG AGT CGG CCG CGA GCG-3'
ZmCKX4bR wo STOP EcoRI
5'-CGG AAT TCC GCG AGT CGG CGA GCG-3'
ZmCKX10 KpnI + Kozak fw
5'-CGG GGT ACC CCA CCA TGA TGC TCG CGT ACA TGG ACC-3'
ZmCKX10R wo STOP EcoRI
5'-CGG AAT TCC GCA CGG CGA CGG ACG CCG-3'
ZmCKX12 KpnI + Kozak fw
5'-CGG GGT ACC CCA CCA TGG AGG GCA AGG TGC TGT G-3'
ZmCKX12R wo STOP EcoRI
5'-CGG AAT TCC GCA TCG GGC TCG AGG AGG AG-3

3.1.5 Plant material and cultivation

- *Arabidopsis thaliana* ecotype Landsberg *erecta* (L*er*) cell suspension culture was provided by Dr. Beata Petrovská from IEB AS CR in Olomouc.
- *Zea mays* cultivar Black Mexican Sweet (BMS) cell suspension cultures provided by Dr. François Chaumont and Dr. Nicolas Richet from Catholic University of Leuven (Belgium).

Cell suspension cultures were grown in 50 mL cultivation media (in 250 mL Erlenmeyer flasks) on the rotary shaker (120 rpm) at 23°C in the dark. Cultures were passaged in 10/7 day intervals by inoculating 10 mL culture into 40 mL of fresh BMS/Ler medium (Tab. 4) with appropriate selection. Cell cultures were also maintained on BMS/Ler medium solidified with 1% Plant Agar as a backup and passaged once per month. All the work with cell suspension cultures was performed under the sterile conditions.

Tab.4: Composition of plant culture media.

BMS medium
4.3 g/L Murashige-Skoog medium (MS). including vitamins
3% sucrose
0.02% L-asparagine
13.5 μM 2.4-dichlorophenoxyethanoic acid (2,4-D)
adjust pH 5.7 (KOH). autoclave

Ler Medium
4.3 g/L Murashige-Skoog medium (MS). including vitamins
3% sucrose
250 nM kinetin
550 nM 2-(1-naphthyl)acetic acid (NAA)
adjust pH 5.7 (KOH). autoclave

3.1.6 Instrumental equipment

Analytical balances model 5034/120 (Auxilab, Spain), Autoclave HST 5-6-8 (Zirbus, Germany), Automatical pipettes (Eppendorf, Germany), Centrifuge Heraeus Megafuge 40 (Thermo scientific, USA, USA), Digital camera (Olympus, Japan), Digital scales Traveller TA302 (Ohaus,Switzerland), Electrophoretic chamber (Biometra, Germany), Electroporator ECM 399 (BTX, USA), Epifluorescent microscope Axio Imager M2 (Zeiss, Germany), Filters Ophthalsart, size of pores 0,2 μm (Sartorius Stedim Biotech, Germany), Gel Documentation system Alpha Digi and software Alpha DigiDoc RT (East Port, Czech Republic), Gel Documentation system ChemiDoc MP (BIO-RAD, USA), Incubated shaker Heidolph unimax 1010 (Heidolph, Germany), Incubator (28 °C) (Lovibond, Germany), Incubator (37 °C) (Medline Scientific limited, Germany), Incubator (65 °C) (Memmert, Germany), Incubator COOL-Hotter Dry (Alex Red, Israel), Laminar Box Faster SCS 2-6 (Ferra, Italy), Laser Scaning Confocal Microscope LSM 710 (Zeiss, Germany), Magnetix stirrer RH basic 2 IKAMAG (IKA, Germany), Micro ultracentrifuge CS150NX (Hitachi Koki, Japan), Mini-PROTEAN 3 Cell (BIO-RAD, USA), pH meter (Eutech Instruments, Singapur), Refrigerated centrifuge Model 5417R (Eppendorf, Germany), Rotary shaker (Kühner, Switzerland), Spectrophotometer NAS 99 (ACT gene, USA), Table microfuge (Biosan, Lithuania), Thermoblock Thermomixer comfort (Eppendorf, Germany), Thermocycler Applied Biosystems Veriti (Life Technologies, USA), Transiluminator UV Superbright (Vilber Lourmat, France), UV/VIS spectrophotometer Agilent 8453 (Agilent, USA).

2.2 Methods

3.2.1 Cloning of ZmCKX-GFP constructs

Expression pGWB5 constructs carrying *ZmCKX1, ZmCKX2, ZmCKX5, ZmCKX6, ZmCKX8* and *ZmCKX9* were prepared within the framework of my former bachelor thesis (Johnová, bachelor thesis, 2013) according to the Gateway cloning technology (Invitrogen, USA). A brief description of individual steps is summarized in following sections.

3.2.1.1 Isolation of plasmid DNA

Plasmid constructs used for cloning of *ZmCKX* genes were isolated using QIAprep Spin Miniprep kit (QIAGEN, Netherlands). Fresh LB medium (Tab. 1) with appropriate antibiotic selection was inoculated from *E. coli* glycerol stock. Overnight culture was harvested by centrifugation, supernatant was removed and pellet was used for plasmid DNA isolation according to the manufacturer protocol. Plasmid DNA was eluted by 20 µl of sterile water. Concentration was measured using NanoDrop Spectrophotometer NAS 99.

In order to achieve higher yields of pLNU-ZmCKX constructs, necessary for maize protoplasttransformation, Nucleobond Xtra Maxiprep (Macherey-Nagel) was used.

3.2.1.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) used for amplification of *ZmCKX* genes was performed with Pfu DNA Polymerase (Fermentas) according to manufacturer protocol. In order to increase amplification efficiency and specificity additives betain and DMSO were added in PCR (Tab. 5, 6). Immolase DNA polymerase (Immomix, Bioline) was used for screening of positive colonies carrying *ZmCKXs* constructs (Tab. 7, 8).

Open reading frames of *ZmCKXs*, obtained from cDNA prepared in previous studies (Šmehilová *et al*., 2009; Zalabák *et al*., 2014), were amplified without a termination codon to allow subsequent in frame fusion with *GFP*. The forward and reverse primers were designed to contain KpnI and EcoRI restriction sites, respectively. In addition, the forward primers were designed to carry the Kozak sequence CCACC, upstream of the initiation codon (Tab. 3).

Tab. 5: Composition of PCR reaction mixture for *Pfu* DNA Polymerase-mediated PCR amplification.

	volume [µL]	Final concentration
10x *Pfu* buffer	2	1x
dNTP mix (10 mM)	0.4	200 µM
4M betaine	6.5	1.3 M
DMSO	0.25	1.3 %
Forward primer (10 µM)	1	0.5 µM
Reverse primer (10 µM)	1	0.5 µM
DNA template (~1 ng/µL)	1	~50 pM
DNA *Pfu* polymerase*	0.2	
Nuclease free H_2O	up to 20	

* Pfu DNA polymerase was provided by Fermentas

Tab. 6: Thermal cycling conditions for *Pfu* DNA Polymerase-mediated PCR amplification.

Step	Temperature	Time	Number of cycles
1. Initial denaturation	95 ºC	5 min	1
2. Denaturation	95 ºC	30 s	
3. Annealing	61 ºC	30 s	35
4. Extension	72 ºC	3 min 3s	
5. Final extension	72 ºC	10 min	1

Tab. 7. Composition of PCR reaction mixture for colony PCR using Immolase DNA polymerase.

	volume [µL]
2x Immomix*	7,5
Fw primer (10 µM)	1
Rev primer (10 µM)	1
DNA Template (bacterial colony)	1
Nuclease free H_2O to final volume of	15

* Immomix was provided by Life Technologies

Tab. 8: Thermal cycling conditions for Immolase DNA polymerase-mediated PCR amplification.

Step	Temperature	Time	Number of cycles
1. Initial denaturation	95 ºC	10 min	1
2. Denaturation	95 ºC	15 s	
3. Annealing	61 ºC	30 s	35
4. Extension	72 ºC	1 min	
5. Final extension	72 ºC	10 min	1

3.2.1.3 Agarose gel electrophoresis of DNA

PCR products or restriction digests were separated in 1% agarose in TAE buffer supplemented with ethidium bromide. Samples were mixed with 6x Loading dye (Thermo scientific, USA) before loading. GeneRuler 1kb plus DNA Ladder (Thermo scientific, USA) was used as a molecular weight standard. DNA fragments were visualized using UV-transiluminator and image was processed by Alpha Digi software.

3.2.1.4 Purification of DNA fragments from agarose gel

DNA fragments were ex*ci*sed from agarose gels with scalpel and extracted using MinElute Gel Extraction kit (QIAGEN, Netherlands) according to the manufacturer protocol and DNA was eluted with 10 µl elution buffer. DNA concentration was determined spectrophotometrically as described above.

3.2.1.5 Preparing of entry clones and pSEG36-EGFP

Firstly, *ZmCKX* genes were subcloned into the plasmid vector pENTR2B d.s., to form so called "entry clone". An entry clone contains gene of interest (*ZmCKX)* flanked by attL1 and attL2 sites, respectively. These attL sequences are then recombined with attR sequences in destination vector (pGWB5) to create desired expression clone pGWB5-ZmCKX see below. PCR amplicons of ZmCKX as well as pENTR2B were digested with KpnI (NEB, USA) and EcoRI-HF (NEB, USA) restriction enzymes (Tab. 9). Digested DNA fragments were purified via Wizard SV Gel or PCR clean-up system kit (Promega, USA) in case of *ZmCKXs*. Afterwards, fragments of *ZmCKX*s genes were ligated into linearized vector using T4 DNA Ligase (NEB, USA; Tab. 10). Ligation mixture was then electroporated into *E.coli* TOP10 cells and plated on LB agar (Kan 50 mg/L, Hyg B 25 mg/L). Positive clones were confirmed using colony PCR, followed by restriction analysis. Selected clones were sequenced to check the *ZmCKX* sequence.

Tab. 9: Restriction reaction of *ZmCKX* genes and pENTR2B vector.

	1 reaction [µL]	
10x NEB 2 buffer	5	
100x BSA	0.5	
KpnI	0.5	1. ON*, 37 ºC
EcoRI-HF	0.3	2. 5h, 37 ºC
Template	~3 µg	
Nuclease free H_2O up to	50	

* Over night

Tab. 10: Ligation of *ZmCKX* genes into pENTR2B vector.

	1 reaction [µL]	Amount of DNA
10x T4 DNA ligase buffer	1	
T4 DNA ligase	1	
Template (~25 ng/µL)	2.6	~65 ng*
Vector (~40 ng/µL)	1.25	~50 ng*
Nuclease free H_2O up to	10	

*Corresponds to the ratio of insert:vector = 2:1.

Plasmid construct pSEG36-EGFP was prepared (Tab., 11, 12) in the same manner as entry vector pENTR2B described above. Restriction enzymes were provided by NEB, USA.

Tab. 11: Restriction reaction of pSEG36-EGFP and pSKM36 vectors.

1 reaction [µL]		
10x Cutsmart buffer	3	
BamHI	0.3	
SpeI	0.5	ON*, 37 ºC
Template	~3 µg	
Nuclease free H_2O up to	30	

* Over night

Tab. 12: Ligation of CaMV35S promotor into pSEG36-EGFP vector.

	1 reaction [µL]	Amount of DNA
10x T4 DNA ligase buffer	1	
T4 DNA ligase	1	
Insert (~50 ng/µL)	0,2	~8 ng*
Vector (~50 ng/µL)	1	~50 ng*
Nuclease free H_2O up to	10	

*Corresponds to the ratio of insert:vector = 2:1.

3.2.1.6 LR recombinant reaction (Gateway technology)

Prepared pENTR2B:*ZmCKX* entry clones were used for LR recombination reaction with a destination vector pGWB5 (Nakawa *et al*., 2007) to create an expression clone pGWB5:*ZmCKX* (Fig. 16). Destination vector pGWB5 (Fig. 16) contains gene encoding for Enhanced Green Fluorescent Protein (EGFP, further in this work named only as GFP), allowing for C-terminal fusion. The recombination reaction is catalyzed by LR clonase II enzyme mixture (Invitrogen, USA; Tab. 13). The reaction mixture was transformed into *E. coli* TOP 10 and screening for positive clones was made by colony PCR in the same way as described above.

Tab. 13: Composition of LR reaction mixture.

	1 reaction
TE buffer pH 8.0	up to 10 µL
Entry clone	50 ng
Destination vector	200 ng -> incubation at 45 ºC, 10 min
LR clonase II	2 µL -> incubation at 25 ºC, ON
Proteinase K	1 µL -> incubation at 37 ºC, 10 min

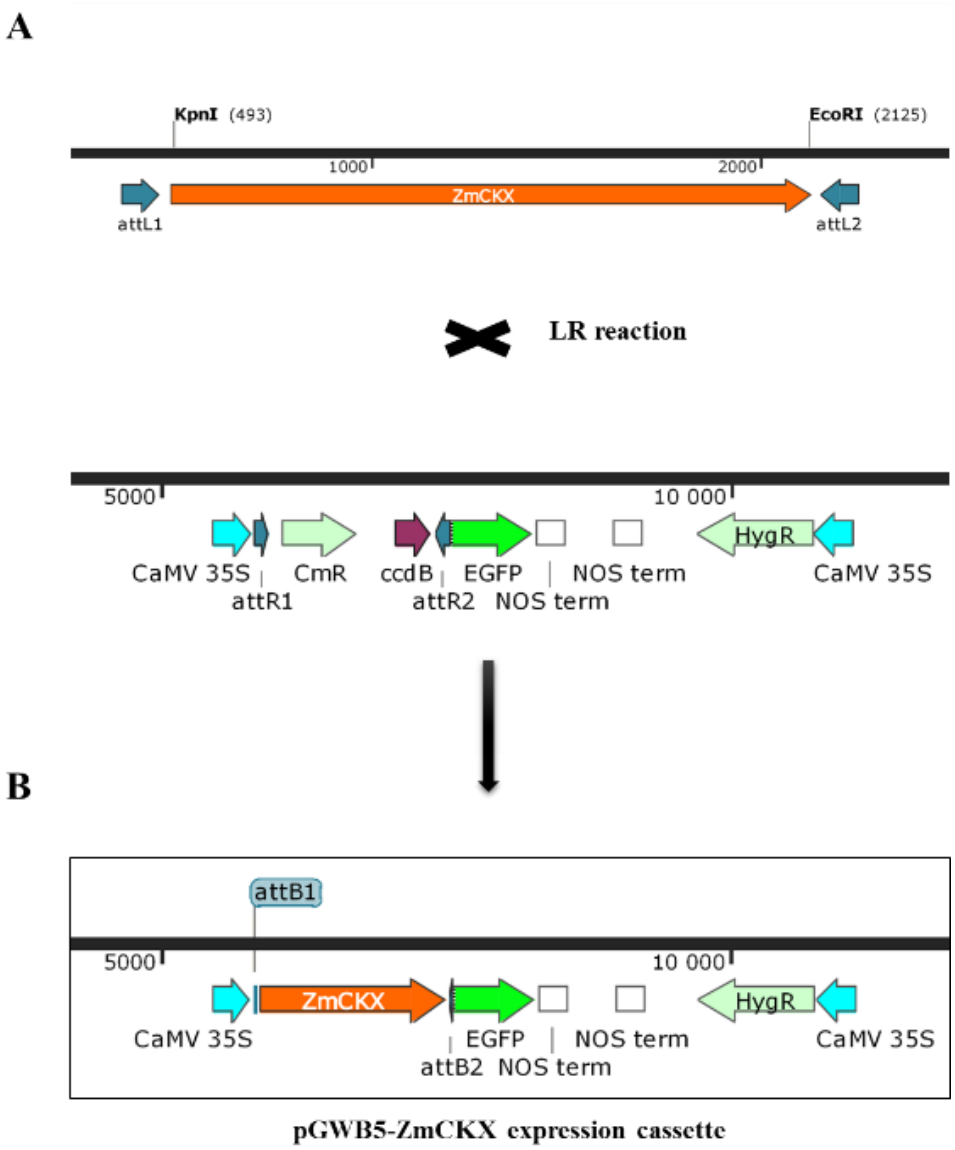

Figure 16: LR recombinant reaction. (A) Entry clone pENTR2B carrying gene of interest *ZmCKX* is recombined with destination vector pGWB5. The result is expression clone containing *ZmCKX-GFP* fusion gene (B).

3.2.1.7 Transformation of electrocompetent bacterial cells

Aliquots of electrocompetent *E. coli* or *A. tumefaciens* cells were thawed on ice and then –about 1 μl of ligation/recombination mixture or plasmid was added to cells and briefly mixed. The mixture was transferred into pre-chilled electroporation cuvette and electroporated at 1800 V for 5 ms. Immediately after electroporation 500 μl of SOC medium was added to the bacterial cells and further cultivated for 1.5 hours at 37 ^{0}C or 28°C, respectively. Bacteria were plated on LB agar including antibiotics and incubated overnight at 37 ^{0}C or 28°C, respectively.

3.2.1.8 Control analysis of plasmid constructs

Positive clones were confirmed using colony PCR and then restriction analysis (Tab. 8). First, colony PCR was mediated using Immolase DNA polymerase and as a templates served picked bacteria colonies from LB agar plates. Samples, which gave PCR product of correct size were used for plasmid isolation and subsequent digestion with restriction enzymes. Optimal conditions for digestion reaction were obtained using Double Digest web tool (www.NEB.com).

3.2.2 Transfection of maize protoplasts

For this purpose, pLNU:*ZmCKX-GFP* plasmid constructs prepared in previous study (Zalabák, 2013, Ph.D. thesis) were co-transformed together with *CaMV35S:AtWAK2-HDEL-tdTomato* construct (ER-tdTomato) into maize protoplasts using polyethylene glycol method. The pLNU:*ZmCKX-GFP* constructs contain C-terminal GFP fusion with ZmCKX . The expression of ZmCKX-GFP fusion proteins is driven by strong constitutive maize Ubiquitin 1 promoter (Ubi1). Construct ER-tdTomato encodes ER marker protein carrying ER retention motif HDEL fused with red fluorescent tag protein tdTomato (tandem dimer Tomato).

Transfection of maize protoplast was performed according to protocol provided by Dr. François Chaumont (Catholic University, Leuven). At first, protoplasts were released from BMS cells using 1.5% Cellulase Y-G and 0.3% Macerozyme R10 (Serva) in digestion buffer. Protoplasts were filtered through 70 μm nylon mesh and then washed twice with digestion buffer without enzymes. Number of protoplast was determined using Burker's counting chamber. Approximately 10, 000 protoplasts were resuspended in 100

μL of transfection medium, and 10–15 μg of plasmid constructs was added. The suspension was carefully mixed with 110 μL of PEG and incubated for 2 min at room temperature (RT). After the addition of 440 μL of W5 buffer, the protoplasts were collected by centrifugation at 100 *g* for 1 min. Harvested protoplasts were resuspended in 1000 μL of digestion buffer without enzymes, and kept overnight in the dark at RT (Tab. 14). Next day, transfected protoplasts were observed using laser scanning confocal microscope.

Tab. 14: Composition of solutions for protoplast transfection.

Digestion buffer	Transfection medium	PEG medium	W5 medium
0.65 M D-sorbitol	0.6 M mannitol	40% PEG 4000	154 mM NaCl
10 mM KCl	8.5 mM $CaCl_2$	0.1 M $Ca(NO_3)_2$	125 mM CaCl2
1 mM $CaCl_2$	5 mM MES	0.3 M mannitol	5 mM KCl
8.7 mM MES	-	-	4 mM MES
pH 5.5	pH 5.7	-	pH 5.7

2.2.3 Transformation of *Arabidopsis thaliana* L*er* suspension cell cultures

The pGWB5:*ZmCKX* constructs were introduced into *A. tumefaciens* GV3101 cells by electroporation. The positive colonies were verified by colony PCR. Agrobacterium-mediated transformation was performed according to protocol adapted from Mathur *et al*. (1998). Briefly, *Agrobacterium* starter culture was prepared by inoculating 2.5 mL of LB media containing appropriate antibiotic selection (25 mg/L gentamycin and 50 mg/L rifampicin for *Agrobacterium* selection, and 50 mg/L kanamycin for pGWB5 vectors) and grown overnight on rotary shaker at 28°C. Next day, the starter culture was used for inoculation of 25 mL of fresh LB media containing antibiotics described above. *Agrobacterium* culture was grown at 28°C until OD_{600} reached value 0.5 – 0.8. The culture was centrifuged at 4000 *g* in precooled centrifuge for 8 min. Pellet was resuspended in 1-2 mL of LER media according to the cell density. *Agrobacterium* culture was activated by 100 μM acetosyringone and further cultivated at RT several hours.

Two days old L*er* suspension cultures were transformed by addition of 200 - 1000 μL of bacterial inoculum into the culture. The transformation was performed in triplicates using various volumes of inoculum. After two days of co-cultivation, the quality of L*er* cells as well as the presence of bacteria was examined under the microscope. When bacteria overgrown, the L*er* cells were harvested by centrifugation at 700 *g* at room

temperature for 2 min and the medium was exchanged. The washing was repeated until the supernatant was clear. At least every second day transformed cultures were examined and washed as required. When the fluorescent signal corresponding to GFP was observed, hygromycin B (25 mg/L) was added to select transformed cells. After about 5 days from starting hygromycin selection, timentin (250 mg/L) was added to remove *Agrobacterium*. During further cultivation transformed culture was pre*ci*sely controlled and concentration of antibiotic was increased up to 50 mg/L of hygromycin B and 500 mg/L of timentin, respectively. One month from transformation transgenic L*er* culture was usually stabilized, and thus passaged once a week. The quality of the cells and the presence of the fluorescence signal were regularly controlled under the epifluorescence microscope.

3.2.4 Isolation of intact vacuoles

Intact vacuoles were isolated from 3-days old *Arabidopsis thaliana* L*er* suspension cells following the protocol of Robert *et al*. (2007).

3.2.5 Microscopic analysis of ZmCKX-GFP fusion proteins

The transformed material carrying ZmCKX-GFP fusion proteins were mounted onto a microscopic slide covered with glass and examined under the Zeiss LSM710 laser scanning confocal microscope with 20x objectives. GFP-tagged proteins and ER-tdTomato marker protein were excited by laser at wavelengths of 488 nm and 561 nm, respectively. Fluorescence emission was detected using a 505/530-nm band pass filter for GFP and 570/590-nm band pass filter for tdTomato. Image processing was performed with Zeiss Zen Black edition software.

3.2.6 Protein extraction from plant material

Suspension cell cultures were harvested using the Büchner funnel and immediately frozen in liquid nitrogen and grinded using mortar and pestle. The flow through culture media (40 mL) was collected into the falcon tube, frozen in liquid nitrogen as well. Protein extraction buffer (Tab. 15) was added to the homogenized plant material in the ratio 1 mL per 1 g of fresh weight. Mixture was kept on ice and extracted for at least 20 min. After the extraction, the sample was 20 min centrifuged at 20 000 g at 4°C. Supernatant was used for subsequent analyses. In contrast, culture media were concentrated by lyophilisation until the powder was formed and then resuspended in 3 mL of extraction buffer. Protein concentration was quantified using Bradford method (Bradford, 1976).

Tab. 15: Composition of protein extraction buffer.

50 mM Tris/HCl, 100 mM NaCl 1mM ethylenediaminetetraacetic acid (EDTA) 0.1% Triton X-100 1 mM phenylmethylsulfonyl fluoride (PMSF) pH = 8

3.2.7 CKX activity assay

The CKX activity in cell lysates and in culture media was measured by end point method (Frébort *et al*., 2002). Aliquots of cell extract (20 to 50 μL) or concentrated media (50 to 100 μL) were incubated in a reaction mixture consisting of 250 mM McIlvaine buffer pH 7.5, 0.125 mM electron acceptor dichlorophenol indophenol (DCPIP) and 250 μM substrate iP. Reaction mixtures (600 μL) were incubated at 37°C for 2-16 h, depending on the enzyme activity. The reaction was terminated by adding 300 μL of 40 % trichloracetic acid (TCA) and 200 μL 2% paraminophenol (PAF) in 6% TCA. After centrifugation, the absorbance of supernatant was measured at 395 nm and specific enzyme activity was calculated according to Frébort *et al*., 2002.

The CKX activity was measured in five days old culture cultivated in the LER media buffered by 20 mM potassium phosphate buffer, pH 6.8.

3.2.8 SDS-PAGE

SDS–PAGE was performed on a 10 % running gel and 4% stacking gel in Tris–glycine running buffer (Tab. 16). The PageRuler Unstained Protein Ladder (Thermo scientific, USA) was used as a marker. Protein samples were mixed with 4x concentrated loading buffer containing (Tab. 16) and boiled at 95 °C for 5 min. Afterwards samples were centrifuged and loaded on the gel. Proteins were separated at 100 V for approximately 20 min and another 30 min at voltage increased to 150 V.

Tab.16: Solutions used for SDS-PAGE.

Acrylamide solution	30% acrylamide 0.8% bisacrylamide	**Electrode buffer**	25 mM Tris. 192 M glycine 0.1% SDS pH = 8.3
Running gel buffer	2.25 M Tris/HCl pH = 9.2	**10 % Running gel**	5 mL of acrylamide solution 2.5 mL running gel buffer 7.26 mL H_2O 0.15 mL 10 % SDS

			0.15 mL TEMED 0.075 mL ammonium persulphate (100 mg/mL)
Stacking gel buffer	0.75 M Tris/HCl pH = 6.8	**4 % Stacking gel**	1 mL acrylamide solution 1.25 mL stacking gel buffer 5.13 mL H_2O 0.075 mL 10 % SDS 0.010 mL TEMED 0.050 mL ammonium persulphate (100 mg/mL)
4x Loading buffer	200 mM Tris-Cl pH 6.8 400 mM DTT 8% SDS 0.4% bromophenol blue 40% glycerol		

3.2.9 Western blotting

Protein transfer from SDS-PAGE gel to polyvinylidene difluoride (PVDF, Thermo scientific, USA) membrane was performed in sandwich-like arrangement: porous pad, filter paper, PVDF membrane activated in methanol, gel and porous pad again. The sandwich was placed into the blotting chamber, overlaid with blotting buffer and electroblotted at 100 V for 1 hour in the fridge. PVDF membrane was stained by amino black solution to visualize protein bands. The colouration was washed in water for 30 min.

Further, PVDF membrane was blocked for 1 h in blocking solution. After the blocking, the membrane was probed with primary antibody (rabbit Anti-GFP antibody (ab290) Abcam, Great Britain; diluted in ration 1:1000 in 1% milk). The membrane was washed once in TBS-T and twice in TBS buffer for 5 min. After that, PVDF membrane was incubated in 1% milk containing secondary antibody (Goat anti-rabbit IgG antibody, Jackson ImmunoResearch Laboratories, USA; diluted in ratio 1:5000) with conjugated horseradish peroxidase. Final washing step was performed in TBS-T for 10 min and twice in TBS buffer, for 10 min each step. Signal was visualized using the Pierce ECL Western blotting Substrate (Thermo scientific, USA, USA) and recorded using system ChemiDoc MP (BioRad) (Tab. 17).

Tab. 17: Western blotting solutions.

Transfer buffer	200 mM Tris/HCl 1.2 M glycine	**Decolorization solution**	40% methanol 10% CH3COOH 50% H2O
Blotting buffer	2.5% (v/v) transfer buffer 20% (v/v) methanol 67.46% (v/v) H_2O 0.04% (w/v) SDS	**TBS washing buffer**	20 mM Tris/HCl 500 mM NaCl pH = 7.5
Colorization solution (membrane)	0.5% (w/v) amide black 50% (v/v) Methanol 7% (v/v) CH_3COOH	**TBS-T**	20 mM Tris/HCl 500 mM NaCl 0.05% (v/v) Tween 20, pH = 7.5
Colorization solution (gel)	0.025% (w/v) Coomassie Brilliant Blue R250 50% methanol 10% CH3COOH	**Blocking buffer**	5% (w/v) milk powder in TBS-T buffer

3 Conclusion

Presented diploma thesis consists of two main parts, theoretical and experimental. In the first theoretical part current knowledge on plant hormones cytokinins is summarised. First part is focused on basic features of cytokinins, cytokinin transport and pointing the main components of cytokinin signal transduction pathway. Last but not least, the most important pathways and enzymes involved in cytokinin metabolism and their interconversions are discussed, with the special stress on cytokinin degradation mediated by cytokinin dehydrogenases.

The intracellular distribution of ten maize cytokinin dehydrogenases isozymes ZmCKX1, ZmCKX2, ZmCKX4a, ZmCKX4b, ZmCKX5, ZmCKX6, ZmCKX8, ZmCKX9, ZmCKX10 and ZmCKX12 was investigated in the frame of the experimental part. For this purpose series of pGWB5 expression constructs containing CaMV35::*ZmCKX1*, *2*, *4a*, *4b*, *5*, *6*, *8*, *9*, 10 and *12* fusions with GFP were prepared partially within the framework of my former bachelor thesis and finished in this thesis (Johnová, bachelor thesis, 2013).

In order to reveal the localization of individual ZmCKX isoforms within the cell, ZmCKX were fused with GFP at the C-termini and expressed. To study the expression pattern of ZmCKX-GFP fusion proteins two different approaches were used. Firstly, ZmCKX-GFP fusion proteins were transiently expressed under the control of constitutive ubiquitin 1 promoter in maize protoplasts using the polyethylene glycol method. For this purpose constructs pLNU:*Ubi1*::ZmCKX*1*, *2*, *4a*, *4b*, *5*, *8*, *9*, *10* and *12* previously prepared (Zalabák, Ph.D. thesis 2013) were used. Subcellular targeting of ZmCKX-GFP fusion proteins in plant cells was analysed via laser scanning confocal microscopy. Transient expression of all the following isoforms ZmCKX1, 2, 4a, 4b, 5, 6, 8, 9 and 12 fused with GFP showed similarly accumulation of GFP signal in the reticular structures, typical for ER. Localization to ER was further confirmed by subsequent co-localization with ER td-Tomato marker. On the contrary, ZmCKX10-GFP fusion protein was found to exhibit bidirectional distribution in cytosol and in nuclei and was therefore comparable with the expression pattern of positive control *Ubi1::GFP*. To verify these results, heterologous expression system in *Arabidopsi*s L*er* cell suspension culture enabling stable expression of C-terminal tagged GFP fusion proteins under the control of constitutive CaMV35S promoter, was established.

Surprising difference in ZmCKX subcellular localization was found among two selected expression systems. While transiently expressed in maize protoplasts, majority of ZmCKX-GFP fusions was localized in ER. Here, the majority of ZmCKX-GFP fusion proteins, namely ZmCKX2, ZmCKX4a, ZmCKX5, ZmCKX8 and ZmCKX12 were targeted to vacuoles. Interestingly, the final destination of ZmCKX9 proteins fusions with GFP was ER. No significant fluorescence was detected in ZmCKX1-GFP expressing cells probably due to its secretion out of the cells. The hypothesis of ZmCKX1 apoplastic localization was proved by CKX activity recorded in culture media. In addition, CKX activity was recorded in cell lysates of all *Arabidopsis* cell suspension lines except ZmCKX6-GFP, which according to former results is supposed to be non-functional. However, in culture media, increased CKX activity was recorded only in the case of ZmCKX1-GFP. Correct cytosolic/nuclear localization of ZmCKX10, similar to free GFP serving as positive control was also proved in this assay. The presence of ZmCKX5, ZmCKX8 and ZmCKX9-GFP fusions was confirmed using Western blotting.

The knowledge on spatial distribution of CKX in monocot plants was fragmented. The only information available were on localization of ZmCKX1 to apoplast and ZmCKX10 to cytosol, respectively (Šmehilová et al., 2009). This study brings the first comprehensive evidence of spatial distribution of cytokinin catabolic enzymes in maize and can therefore be generalized to other monocot species. Compared to *Arabidopsis*, more CKX isozymes are involved in controlling cytokinin homeostasis in maize. Whereas most of the ZmCKX isozymes act in one compartment – vacuoles, there is only one isoform ZmCKX1 secreted out of the cell which is considered to play a key role in regulation of cytokinin levels in apoplastic space. To conclude, difference in subcellular localization suggests that specific developmental and physiological functions are performed by each CKX isozyme and thus the tissue-specific regulation as well as regulation on subcellular level of the endogenous cytokinin content is an important prerequisite in the regulation of cytokinin functions *in planta*.

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5 Abbreviations

ADK	adenosin kinase
ADP	adenosine diphosphate
AHK	*Arabidopsis* histidine kinase
AHP	*Arabidopsis* histidine-containing phosphotransfer proteins
AMP	adenosine monophosphate
ARR	*Arabidopsis* responsive regulators
ATP	adenosine triphosphate
AtPUP	*Arabidopsis* purine permeases
BA	benzylaminopurine
CK	cytokinin
CKX	cytokinin dehydrogenase
CRF	cytokinin response factors
*c*Z	*cis*-zeatin
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
DZ	dihydroxyzeatin
EGFP	enhanced GFP
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide
GFP	green fluorescent protein
GUS	β-glucuronidase reporter gene
HMBDP	(E)-4-hydroxy-3-methyl-but-2-enyl
CHASE	cyclases/histidine kinases associated sensor extracellular)
iP	isopentenyladenine (
IPT	isopentenyltransferase
LOG	Lonely guy, cytokinin riboside 5-monophosphate phosphoribohydrolase
MEP	methylerythritol phosphate
MVA	mevalonate
RAM	root apical meristem
SAM	shoot apical meristem
tRNA	transfer ribonucleic acid
*t*Z	*trans*-zeatin
ZmCKX	*Zea mays* cytokinin dehydrogenase