# PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Department of Biochemistry


# Cytokinins as mediators of plant physiological responses

Ph.D. Thesis


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Abstrakt

Práce je zaměřena na metabolismus rostlinných hormonů cytokininů a je rozdělena do dvou částí. První část se věnuje ověření předpokládané lokalizace nesekretované isoformy CKX z kukuřice (ns-ZmCKX) a objasnění její funkce na základě porovnání s apoplastickou ZmCKX1. Byl nově naklonován gen pro ns-ZmCKX, který byl anotován jako *ZmCKX10* (GenBank no. FJ269181). Použitím GFP fúze s následnou overexpresí v pletivech kořene rajčete byla pomocí konfokální mikroskopie ověřena jeho subcelulární lokalizace v cytosolu. Pro biochemickou charakterizaci byl připraven rekombinantní ZmCKX10 v expresním systému *Pichia pastoris*. ZmCKX10 byl pomocí MALDI-TOF/MS identifikován jako 56,3 kDa protein a byla stanovena jeho preference pro elektronové akceptory a substrátová specifita. V neposlední řadě byla popsána změna fenotypu kořenů rajčete overexprimujících *ZmCKX10-GFP* a *ZmCKX1-GFP*.

Druhá část této práce se zabývá rolí cytokininů v odpovědi na stres a v průběhu senescence. Pomocí qPCR byl stanoven expresní profil genů metabolismu cytokininů v průběhu přirozené senescence listu *A. thaliana.* Byla též stanovena aktivita CKX a obsah cytokininů v těchto listech. V rámci objasnění regulace procesů senescence cytokininy byly dále testovány nově syntetizované deriváty cytokininů s předpokládanými antisenescenčními účinky. Byly pozorovány rozdíly v účincích těchto látek na míru hladin sledovaných transkriptů u listů *A. thaliana* a kukuřice. V neposlední řadě byl sledován vliv testovaných látek na fenotyp obou rostlin.

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Abstract

This work is focused on metabolism of plant hormones cytokinins and consists of two parts. In the first part, verification of predicted subcellular localization of a maize non-secreted CKX isoform (ns-ZmCKX) is described in comparison to the apoplastic ZmCKX1. The *ns-ZmCKX* gene (annotated as *ZmCKX10*, GenBank no. FJ269181) cloning is included. Further, its predicted subcellular localization is confirmed to the cytosol using confocal microscopy of tomato hairy roots overexpressing *ZmCKX10* fused with GFP. For biochemical characterization, the ZmCKX10 recombinant protein was prepared using *Pichia pastoris* expression system. ZmCKX10 was identified as a 56.3 kDa protein using MALDI-TOF/MS and subsequently, electron acceptor preference and substrate specificity was determined. Finally, phenotypical changes of tomato hairy roots overexpressing *ZmCKX10-GFP* and *ZmCKX1-GFP* were described.

The second part of this work deals with the cytokinins' role in plant stress responses and senescence. Quantitative analysis of the relative transcript levels was used to determine expression profiles of the genes involved in cytokinin metabolism and perception during natural leaf senescence in *Arabidopsis*. A CKX activity assay was performed and cytokinin content was determined in senescent *Arabidopsis* leaves. Novel cytokinin derivatives with proposed anti-senescence activity were examined for their effects to delay senescence in *Arabidopsis* and maize leaves in terms of gene expressions modulation detection and phenotype changes determination.

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## Table of Content

Souhrn

Práce je zaměřena na metabolismus rostlinných hormonů cytokininů. Ty slouží v rostlinných buňkách jako důležité signální molekuly kontrolující buněčné dělení, apikální dominanci, senescenci, vývoj plodů a další. Pro pochopení mechanismu signalizace cytokininů je důležité porozumět jejich metabolismu a charakterizovat aktivity enzymů do něj zapojených v jednotlivých buňkách a pletivech, potažmo regulovat exprese genů tyto enzymy kódující. Regulace homeostase cytokininů sestává z jejich biosyntézy, aktivace, deaktivace, percepce a transportu. Jedním z esenciálních způsobů regulace cytokininů je jejich deaktivace enzymy cytokinin dehydrogenasami (CKX; EC 1.5.99.12), které tyto fytohormony ireversibilně degradují. CKX jsou ve vyšších rostlinách kódovány malými genovými rodinami čítajícími několik členů. Jednotlivé isoformy jsou v buňkách rozdílně cíleny tak, aby mohly zastávat své specifické regulační funkce. Specifické prostředí buněčných kompartmentů předurčuje odlišné biochemické vlastnosti jednotlivých isoforem CKX a ty následně hladiny aktivních cytokininů v jednotlivých kompartmentech buňky. Většina isoforem CKX ve svých sekvencích obsahuje třídící signální peptid, který jednotlivé isoformy směřuje do apoplastu či vakuol. U většiny sledovaných rostlin bylo zjištěno, že ze všech isoforem CKX má každá rostlina pouze jednu, která sekreční signál postrádá a tudíž by měla být exprimována do cytosolu. U takové isoformy se předpokládají odlišné vlastnosti, dané prostředím cytosolu. Zatímco buněčná lokalizace a biochemická charakterizace mnoha sekretovaných isoforem CKX již byla experimentálně ověřena, málo bylo známo o isoformách nesekretovaných.

Cílem první části této práce proto bylo ověření předpokládané lokalizaci nesekretované isoformy CKX z kukuřice (ns-ZmCKX) a objasnit její funkci a to vše na základě porovnání s isoformou apoplastickou (již dříve charakterizovanou), rovněž z kukuřice (ZmCKX1). Za tímto účelem byl nově naklonován gen pro ns-ZmCKX, který byl později anotován jako *ZmCKX10* (GenBank no. FJ269181). Pro studium subcelulární lokalizace bylo použito značení zeleným fluorescenčním proteinem (GFP) a následná overexprese fúzních proteinů v pletivu kořenů rajčete pomocí tzv.: „Tomato hairy root tansformation“. Použitím konfokální mikroskopie bylo zjištěno, že ZmCKX10-GFP je exprimován do cytosolu, kdežto ZmCKX1-GFP byl potvrzen v apoplastu. Stanovením aktivity CKX byla potvrzena funkčnost obou fúzních proteinů, kdy oba prokazatelně zvyšovaly CKX aktivitu kořenů rajčete v porovnání s kontrolu. V neposlední řadě byla pozorována a popsána změna fenotypu kořenů rajčete overexprimujících *ZmCKX1-GFP* a *ZmCKX10-GFP*. Zatímco u kořenů exprimujících apoplastickou isoformu byl detekován nárůst biomasy, kořeny s overexprimovanou ZmCKX10-GFP tvořily biomasy o něco méně ve srovnání s kontrolu. Pro biochemickou charakterizaci byl připraven rekombinantní ZmCKX10 pomocí expresního systému kvasinek *Pichia pastoris*. ZmCKX10 byl částečně purifikován a poté pomocí MALDI-TOF/MS identifikován jako 56.3 kDa protein o 525 aminokyselinách. Byla stanovena jeho preference pro elektronové akceptory a substrátová specifita, která se zásadně lišila ve srovnání s ZmCKX1.

ZmCKX10 preferuje *cis*-zeatin a *N*-glukosidy a jeho aktivita je ovlivnitelná v podobné míře různými elektronovými akceptory.

Jak již bylo zmíněno a jak naznačují výsledky experimentů popsaných výše, jednotlivé isoformy CKX zastávají v jednotlivých kompartmentech rostlinných buněk rozdílné specifické funkce. Experimentálně bylo prokázáno, že jsou velmi specificky místně a časově exprimovány a různým způsobem reagují také při odpovědi rostlin na stres. Na modelových rostlinách *Arabidopsis thaliana* bylo zjištěno, že během senescence se v listech zásadně zvedají hladiny některých těchto isoforem a to konkrétně apoplastických AtCKX2 a AtCKX5. Druhá část této práce se proto zabývá rolí cytokininů v odpovědi na stres a v průběhu procesu senescence. Pro hlubší pochopení role cytokininů v senescenci byly u různě starých listů *A. thaliana* pomocí kvantitativní „Real-time" PCR stanoveny expresní profily vybraných genů podílejících se na metabolismu a percepci cytokininů a některých dalších genů zapojených do senescence. Dále byla též stanovena CKX aktivita a obsah cytokininů v senescenčních listech *A. thaliana*. Tyto experimenty prokázaly zvýšenou aktivaci CKX jak na úrovni transkripce, tak na úrovni aktivního proteinu. Mimo to bylo zjištěno například to, že během přirozené senescence v listech jsou různě modulované geny pro aktivaci, deaktivaci a percepci cytokininů, zatímco exprese genů pro biosyntézu zůstaly nezměněny. V rámci objasnění regulace procesů probíhajících během senescence vlivem cytokininů byly dále testovány i některé nově syntetizované deriváty cytokininů s předpovězenými antisenescenčními účinky. Konkrétně byly pozorovány rozdíly v účincích různých $N^9$-substitučních derivátů kinetinu na míru exprese genů zapojených do metabolismu cytokininů a některých genů asociovaných se senescencí na ustřižených listech i na listech z intaktních rostlin *A. thaliana* a *Zea mays* inkubovaných ve tmě nebo během fotoperiody. Byly prokázány antisenescenční účinky těchto derivátů se silnějším efektem za tmy a to jak při přímém příjmu listy, tak skrze kořeny. Antisenescenční účinky $N^9$-2-chlorethylového substitučního derivátu kinetinu 6FA9ClEP byly pozorovány pouze u kukuřice, přičemž u *Arabidopsis* nebyl tento derivát vnímán vůbec. V neposlední řadě byly popsány pozorované změny fenotypu vyvolané působením testovaných derivátů cytokininů a byl předpovězen jejich antistresový účinek. Zjištěné vlastnosti testovaných derivátů naznačují jejich případné využití v biotechnologii zemědělky využitelných plodin. Výsledky experimentů popsaných v této práci přispěly ke komplexnějšímu objasnění role cytokininů v senescenci u rostlin a zapojení jednotlivých isoforem CKX do regulace tohoto procesu.

**Summary**

This work is focused on metabolism of plant hormones cytokinins (CKs). These phytohormones play their role as signal molecules in plant cells. They control cell differentiation, apical dominance, senescence, fruit development and many other processes. To understand the mechanism of cytokinin signalization, it is necessary to comprehend also cytokinin metabolism and its precise equilibrium by up- or down-regulation of genes involved in all metabolic activities within plant cells. Cytokinin homeostasis consists of biosynthesis, activation, inactivation, perception and transport. One of the essential mechanisms of CKs regulation is their inactivation by the cytokinin dehydrogenase enzyme (CKX; EC 1.5.99.12) that irreversibly degrades their molecules. These CKX enzymes are encoded by small gene families counting several members in each plant species. There are several isoforms of CKX in plants that are targeted differently within cell compartments. Whereas most of the isoforms are secreted to apoplast or targeted to vacuoles, there is generally one unique isoform per plant species that lacks secretion signal and therefore should be expressed to cytosol. Different biochemical properties were suggested for a non-secreted isoform given by cytosolic environment. Whilst many targeted CKX isoforms have been already biochemically characterized and confirmed for their subcellular localization, little was known about non-secreted forms.

Thus, the first part of this work aims to confirm predicted cytosolic localization of a potentially non-secreted CKX isoform from maize (ns-ZmCKX) and moreover, to elucidate its function in comparison to apoplastic ZmCKX1. For this purpose, a novel gene for ns-ZmCKX was amplified, cloned and annotated as *ZmCKX10* (GenBank no. FJ269181). The gene was fused to GFP and overexpressed in tomato hairy roots to confirm predicted subcellular localization. Using confocal microscopy, ZmCKX10-GFP was revealed to be localized to cytosol while ZmCKX1-GFP was confirmed to apoplast. A CKX activity assay of the tissues overexpressing fusion proteins was used to verify functionality of both enzymes. Besides this, phenotypes of tomato hairy roots overexpressing *ZmCKX10-GFP* and *ZmCKX1-GFP* were described. The roots overexpressing apoplastic CKX isoform showed enhanced biomass formation in contrast to a small reduction caused by overexpressed ZmCKX10-GFP. Both overexpressed GFP fused proteins enhanced the CKX activity in tomato hairy roots in comparison with the control. Furthermore, ZmCKX10 was prepared in *Pichia pastoris* expression system for further biochemical characterization. The recombinant ZmCKX10 was identified as 56.3 kDa protein using MALDI-TOF/MS. Its electron acceptor preference and substrate specificity was determined. To conclude the results of the first part, ZmCKX10 was found to have a higher preference for *cis*-zeatin and *N*-glucosides as substrates with a range of suitable electron acceptors.

As stated and as the results of the experiments described above suggest, distinct CKX isoforms maintain specific functions in different subcellular compartments. It is generally known, that

CKX isoforms are expressed with spatial and temporal specificity. Furthermore, CKX isoforms were revealed to reflect plant stress responses. Hence, experiments on *Arabidopsis* had shown up massive up-regulation of apoplastic AtCKX2 and AtCKX5 during senescence. Second part of this work is focused on cytokinins as mediators of plant stress responses and senescence. A quantitative Real-time PCR analysis was employed to determine an expression profile of the genes involved in cytokinin metabolism and perception in relation to natural senescence in *Arabidopsis*. Furthermore, CKX activity was assessed and cytokinin content was determined in senescent leaves of *Arabidopsis*. These experiments proved enhanced CKX action on the transcriptional level and also on the level of active protein. Besides this, varying modulation of genes for cytokinin activation, deactivation and perception was observed, whereas the biosynthetic pathway stayed unaffected during leaf senescence. Moreover, to elucidate the cytokinin regulation of senescence process, novel cytokinin derivatives with a proposed anti-senescence activity were tested. Specifically, distinct $N^9$-substiuted derivatives of kinetin were analysed for their impact on cytokinin metabolism and senescence associated gene expressions modulation. These experiments were performed on *Arabidopsis* and *Zea mays* leaves or leaves from intact plants under different light conditions. The anti-senescence effects of the kinetin derivatives were proved when receiving directly by leaves or by roots. However, anti-senescent effect of $N^9$-2-chloroethyl substituted kinetin derivative 6FA9ClEP was observed only on maize. Finally, phenotypes of cytokinin derivatives treated plants were assessed and possible anti-stress effects were predicted. The results of these experiments suggest possible applications of novel $N^9$-substituted cytokinin derivatives in senescence regulation in crop biotechnology. Taken together, the presented work contributes to the general understanding of the cytokinins' role in senescence and involvement of CKX isoforms in regulation of this process.

**Aims of the Work**

1. Subcellular localization and biochemical comparison of cytosolic and secreted cytokinin dehydrogenase enzymes from maize:
    - To isolate a gene for non-secreted cytokinin dehydrogenase from maize (*ZmCKX10*);
    - To elucidate the subcellular localization of ZmCKX10 in comparison to apoplastic ZmCKX1;
    - To specify phenotypes of plant tissues overexpressing *ZmCKX10* and *ZmCKX1*;
    - To characterize the ZmCKX10 protein.
2. Cytokinin metabolism gene expressions profiles in *Arabidopsis* leaf during senescence: response to kinetin $N^9$-substituted derivatives:
    - To assign expression profiles of the genes involved in cytokinin metabolism and perception during leaf senescence of *Arabidopsis thaliana*;
    - To perform a cytokinin dehydrogenase (CKX) assay and to determine CKs content in *A. thaliana* leaves during senescence;
    - To analyze responses to cytokinin derivatives on expression profiles of cytokinin metabolism involved genes of *A. thaliana leaves*.

# Part 1

## Subcellular localization and biochemical comparison of cytosolic and secreted cytokinin dehydrogenase enzymes from maize

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**Abstract**

Cytokinin dehydrogenase (CKX; EC 1.5.99.12) degrades cytokinin hormones in plants. There are several differently targeted isoforms of CKX in plant cells. While most CKX enzymes appear to be localized in the apoplast or vacuoles, there is generally only one CKX per plant genome that lacks a translocation signal and presumably functions in the cytosol. The only extensively characterized maize CKX is the apoplastic ZmCKX1; a maize gene encoding a non-secreted CKX has not previously been cloned or characterized. Thus, the aim of this work was to characterize the maize non-secreted CKX gene (*ZmCKX10*), elucidate the subcellular localization of ZmCKX10, and compare its biochemical properties with those of ZmCKX1. Expression profiling of *ZmCKX1* and *ZmCKX10* was performed in maize tissues to determine their transcript abundance and organ-specific expression. For determination of the subcellular localization, the CKX genes were fused with green fluorescent protein (GFP) and overexpressed in tomato hairy roots. Using confocal microscopy, the ZmCKX1–GFP signal was confirmed to be present in the apoplast, whereas ZmCKX10–GFP was detected in the cytosol. No interactions of ZmCKX1 with the plasma membrane were observed. While roots overexpressing *ZmCKX1–GFP* formed significantly more mass in comparison with the control, non-secreted CKX overexpression resulted in a small reduction in root mass accumulation. Biochemical characterization of ZmCKX10 was performed using recombinant protein produced in *Pichia pastoris*. In contrast to the preference for 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor and *trans*-zeatin, $N^6$-($\Delta^2$- isopentenyl)adenine (iP) and $N^6$-($\Delta^2$-isopentenyl)adenosine (iPR) as substrates for ZmCKX1, the non-secreted ZmCKX10 had a range of suitable electron acceptors, and the enzyme had a higher preference for *cis*-zeatin and cytokinin *N*-glucosides as substrates.

**Key words:** Apoplast, CKX, CKX activity assay, cytokinin, cytosol, GFP, *Pichia pastoris* expression system, subcellular localization, tomato hairy root transformation, *Zea mays*.

## Introduction

Cytokinins are phytohormones that serve as important signaling molecules in plant cells. As hormones, they control cell division and influence apical dominance, leaf senescence, fruit development and other processes (Mok and Mok, 2001). To find out how cytokinins carry out signaling functions, it is critical to understand the regulation of cytokinin homeostasis and activity in plant tissues. Cytokinin dehydrogenases (CKXs; EC 1.5.99.12) play an essential role in cytokinin regulation through irreversible degradation of the phytohormone. They inactivate the hormone molecule by cleavage of the isoprenoid side chain of *trans*-zeatin, $N^6$-($\Delta^2$-isopentenyl)adenine, and their ribosides to produce adenine or adenosine and the corresponding aldehydes (for a review see Popelková *et al.*, 2004). A *CKX* gene was first cloned from maize by Morris and co-workers (Morris *et al.*, 1999) and simultaneously by the team of Houba-Hérin (Houba-Hérin *et al.*, 1999). Shortly afterwards, it became apparent that plants contain small families of these genes. To date, such gene families have been identified and characterized in varying degrees in maize, *A. thaliana*, rice, wheat, and barley (Bilyeu *et al.*, 2001; Bilyeu *et al.*, 2003; Galuszka *et al.*, 2004; Massonneau *et al.*, 2004; Popelková *et al.*, 2004). Sequence analysis of the first cloned *CKX* gene from maize (*ZmCKX1*, GenBank accession no. NM_001112121) revealed an N-terminal signal peptide that was predicted to target the enzyme to the cell secretory pathway (Morris *et al.*, 1999). Identification of glycosylation sites in the protein was also consistent with this prediction. Indeed, recombinant production of ZmCKX1 with its native signal peptide resulted in secretion from the yeast, *Pichia pastoris*, and from moss protoplasts (Houba-Hérin *et al.*, 1999; Morris *et al.*, 1999; Bilyeu *et al.*, 2001). The structure of the glycosylated protein has been determined recently (Malito *et al.* 2004). CKX enzymes are oxidoreductases that can catalyze the degradation of cytokinins with molecular oxygen as an electron acceptor (oxidase reaction) or with other electron acceptors in a dehydrogenase reaction (Galuszka *et al.*, 2001; Laskey *et al.*, 2003; Frébortová *et al.*, 2004). For example, in our studies of ZmCKX1, we demonstrated the potential of either reaction pathway (Frébortová *et al.*, 2004). Overexpression of CKX-encoding genes typically resulted in a cytokinin deficiency phenotype showing retarded stem growth and excessive root system development (Werner *et al.*, 2003; Galuszka *et al.*, 2004).

In further studies using immunohistochemical methods to detect native ZmCKX1 in maize tissues, we localized this enzyme to the apoplast region (Galuszka *et al.*, 2005). The extensive characterization of the membrane association of ZmCKX1 was prevented due to relatively low expression in maize tissues and lack of the antibody specific to ZmCKX1 (not cross-reacting with other CKX isozymes). In all plants with sufficient sequence data for analysis, targeted forms of *CKX* occur as multiple gene copies. Another maize CKX enzyme (CKO3) was secreted to the culture media when expressed with its native signal sequence in a heterologous *Yarrowia lipolytica* expression system (Massonneau *et al.*, 2004). The fluorescence signals of two *Arabidopsis* CKX proteins fused

with the green fluorescent protein (GFP) tag were detected in vacuoles in root cells as well as in leaf cells (Werner *et al.*, 2003). Non-targeted variants of *CKX* genes (lacking predicted N-terminal signal peptide sequences) have also been identified *in silico* in several plant species (*Oryza sativa*, *Glycine max*, *Hordeum vulgare*, and *Triticum aestivum*; unpublished results). In *A. thaliana*, a single copy of the only non-targeted CKX, AtCKX7, was functionally characterized (Galuszka *et al*., 2007).

Both targeted and non-targeted CKX enzymes are flavin-associated proteins (Bilyeu *et al.*, 2001). The transfer of electrons within the plant becomes an intriguing issue for targeted and non-targeted enzymes that may be localized to distinct redox environments within compartmentalized cells and tissues. Constitutive overexpression of the CKX genes proved to be a valuable tool in assessing physiological role of cytokinins in model plants (Werner *et al.*, 2001). Since only the targeted CKX enzymes were studied so far, it is important to investigate and compare the outcome of overexpression of cytosolic enzyme.

In this work, the non-secreted *CKX* gene from maize *(ZmCKX10*) was isolated using the sequence information obtained from a survey of the maize genome database. Using confocal microscopy, the subcellular destinations of GFP fusions of ZmCKX10 and ZmCKX1 were compared in the tomato hairy root expression system.

## Materials and methods

*Isolation of ZmCKX10 gene*

RNA was isolated from *Zea mays* cv. Cellux 225 kernels 2-3 weeks after pollination using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was carried out using 5 µg of total RNA with the RevertAid™ H Minus M-MuLV Reverse Transcriptase protocol (Fermentas, Vilnius, Lithuania). Specific primers ns_f 5´-ATGATGCTCGCGTACATGGACC-3´ and ns_r 5´-TTGCTCTACACGGCGACGGA-3´ were designed. The gene for ZmCKX10 was amplified using Phusion DNA polymerase (Finnzymes, Espoo, Finland) with addition of 1.3% DMSO and 1.3 M betaine (Henke *et al.*, 1997). A TGradient Thermocycler (Biometra, Goettingen, Germany) was used for thermal cycling as follows: 3 min at 96°C, followed by 45 cycles of 20 s at 96°C, 45 s at 61°C, 60 s at 72°C; and terminated by 10 min at 72°C. The full-length cDNA was cloned into pDrive vector using the Qiagen PCR Cloning Kit (Qiagen, Valencia, CA, USA). A plasmid DNA was isolated from the selected clones using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced (DNA Core, Bond Life Sciences Center, University of Missouri, Columbia MO; Core Laboratory, Department of Functional Genomics and Proteomics, Masaryk University, Brno, Czech Republic).

*Site-directed mutagenesis of ZmCKX10*

The ORF for *ZmCKX10* was repaired in two positions using the QuikChange Site-Directed Mutagenesis Kit using the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Primers were designed according to the QuikChange® Primer Design Program (Stratagene). For ATC to GTC change at position 376 (Ile to Val) the following primers were used: forward a376g_f 5'-GCTTCGCCGACGTCCCCGGCGGC-3' and reverse a376g_r 5'-GCCGCCGGGGACGTCGGCGAAGC-3'. For the AAG to CAG change at position 688 (Lys to Gln) a688c_f 5'-ACAGGGCGCCCCAGGCGGTGCGG-3' and a688c_r 5'-CCGCACCGCCTGGGGCGCCCTGT-3' were used, respectively. As a template, 50 ng of vector DNA was used per reaction. The *ZmCKX10* was repaired in two separate reactions each in 12 cycles with annealing at 68°C. Mutated DNAs were analysed by sequencing.

*Quantitative PCR analysis*

Total RNA for reverse transcription was isolated using RNAqueous® kit and Plant RNA Isolation Aid solutions (Applied Biosystems/Ambion, Austin, TX, USA). Isolated RNA was treated by TURBO DNase-free™ kit (Ambion) to minimize the bias in qPCR data caused by traces of genomic DNA contamination. First strand cDNA was synthesized by RevertAid™ H Minus M-MuLV RT and random hexamer primers (Fermentas). Diluted cDNA samples were used as templates in real-time

PCR reactions containing POWER SYBR® Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) and 300 to 900 nM of forward and reverse primers. The primers were designed using Primer Express 3.0 software (Applied Biosystems). RNA from each tissue was transcribed in two independent reactions and each cDNA sample was run in at least two technical replicates on StepOnePlus™ Real-Time PCR System in a default program (Applied Biosystems). The specificity of the amplification was verified by melting curve analysis. For both genes, plasmid DNA containing the gene was used as a template to generate calibration curve to determine the efficiency of PCR reactions. The 18S small subunit ribosomal rRNA gene (GenBank accession no. AF168884) was used as the endogenous control to normalize the variations in quality of RNA and efficiency of transcription among the samples. The sequences of the applied primers were designed as follow: 18S_f 5´-CCATCCCTCCGTAGTTAGCTTCT-3´ and 18S_r 5´-CCTGTCGGCCAAGGCTATATAC-3´ for 18S RNA, Zm1_f 5´-TGCAGGGCACCGACATC-3´ and Zm1_r 5´-CGTCCCACATGGATTTGTTG-3´ for *ZmCKX1* (reverse primer overlaps exon2/3 junction), Zm10_f 5´-CGACATCGCCGACTTCGA-3´ and Zm10_r 5´-GGGTCCCACTTGCTCTTGAG -3´ for *ZmCKX10* (reverse primer overlaps exon3/4 junction). For both genes, plasmid DNA containing the gene was used as a template to generate a calibration curve to determine the efficiency of PCRs. The efficiency of amplification was calculated from standard curves done for 102–109 gene copy numbers as 99.2% ($R^2$=0.998) for ZmCKX1 and 101.2% ($R^2$=0.996) for ZmCKX10, respectively. The copy number was calculated for 1 ng of mRNA sample isolated from embryo, which was used as a calibrator for ΔΔCt relative quantification methods corrected by an efficiency factor.

*Production and purification of recombinant ZmCKX10*

To prepare recombinant ZmCKX10, expression in *Pichia pastoris* X-33 (Invitrogen) was used. The full length cDNA of *ZmCKX10* gene was cloned to pPICZA *Pichia pastoris* expression vector (Invitrogen) downstream of the AOX1 inducible promoter.

pPICZA:*ZmCKX10* transformants were obtained using transformation of *P. pastoris* with linearized pPICZA:*ZmCKX10* vector by electroporation following the manufacturer's protocol (Invitrogen). The transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing 100 mg $l^{-1}$ zeocin (Duchefa Biochemie B.V., Haarlem, The Netherlands). Selected transformants were analysed for plasmid integration using PCR according to manufacturer's protocol (Invitrogen). Selected clones were grown overnight in 50 mL of BMGY media consisting of 1% yeast extract, 2% peptone, 1.34% Yeast Nitrogen Base w/o Amino Acids (YNB, Difco Laboratories, Detroit, MI, USA), 4 x $10^{-5}$% d-biotin, 1% glycerol, buffered to pH 6.5 by $K^+$ phosphate buffer at 30°C with extensive shaking at 230 rpm. The overnight culture was centrifuged at 1500 x g for 5 min, washed and resuspended to $OD_{600}$ = 1 in BMMY media (1% yeast extract, 2% peptone, 1.34% YNB (Difco), 4 x $10^{-5}$% d-biotin) buffered with $K^+$ phosphate buffer pH 6.5. The

expression was induced by addition of methanol to a final concentration of 0.5%, and this concentration was maintained for three days. After 3 days, the cells were harvested by centrifugation at 1500 x g for 5 min at 4°C. The cells were broken using 0.45 mm glass beads (Sigma-Aldrich, St. Louis, MO, USA) in a breaking buffer (50 mM sodium phosphate pH = 7.4, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM EDTA, 5% glycerol). The lysate was concentrated using ultrafiltration on a Microcon YM-10 membrane (cut-off 10 kDa, Millipore, Billerica, MA, USA) and kept at 4°C in 0.05 M Tris-HCl pH 8.0. The concentrated proteins were loaded on an octyl-Sepharose CL-4B hydrophobic column (GE-Healthcare, Freiburg, Germany) connected to a BioLogic LP liquid chromatograph system (Bio-Rad, Hercules, CA, USA). After applying the sample, the column was washed with a descending step-gradient of ammonium sulphate, and the eluate was fractionated. The fractions showing enzyme activity were pooled and concentrated using a stirred ultrafiltration cell (Millipore, Bedford, MA, USA) equipped with the YM-10 membrane. The purified ZmCKX10 was analysed using a Microflex MALDI-TOF LR20 mass spectrometer equipped with a nitrogen laser (Bruker Daltonic, Bremen, Germany) following a published protocol (Pospíšilová *et al*., 2008).

*CKX activity assay*

The activity was measured using a modified end-point method described earlier (Frébort *et al.*, 2002). For activity screening, the samples were incubated in a reaction mixture (total volume of 0.6 mL in 1.5 mL tube) that consisted of 200 mM McIlvaine buffer (100 mM citric acid and 200 mM $Na_2HPO_4$) pH 6.5, an electron acceptor and a substrate. For basic measurement, 500 µM 2,6-dichlorophenol indophenol (DCPIP; LOBA Feinchemie, Fischamend, Austria) and 250 µM $N^6$-($\Delta^2$-isopentenyl)adenine (iP, Sigma-Aldrich) as the substrates were used. The volume of the enzyme sample used for the assay was adjusted based on the enzyme activity. Incubation time at 37°C was 1-16 hours.

The enzymatic reaction was stopped after incubation by adding 0.3 mL of 40% trichloroacetic acid, and the sample was centrifuged at 19,500 x *g* for 5 min to remove protein precipitate. After that, 0.2 mL of 4-aminophenol (2% solution in 6% trichloroacetic acid) was added to the supernatant followed by immediate (within 3 min) scanning of the absorption spectrum from 300 to 700 nm to determine the concentration of produced Schiff base with $\varepsilon_{352} = 15.2\ mM^{-1}\ cm^{-1}$ (Frébort *et al.*, 2002).

For determination of electron acceptor preference of ZmCKX1 (enzyme as described: Bilyeu *et al.*, 2001) and ZmCKX10 (produced as described above), the enzymatic activity was assayed with the following electron acceptors: 100 µM DCPIP, 250 µM $Q_0$, 250 µM ferricyanide (Lachema, Brno, Czech Republic) or without the acceptor (in presence of oxygen). For the assessment of substrate specificity, a modified continual method for CKX activity measurement (Laskey *et al.*, 2003) was

used with 100 μM DCPIP and a variety of substrates (50 μM): iP and isopentenyladenine 9-glucoside (iP9G) (both from Sigma-Aldrich) and isopentenyladenosine (iPR), isopentenyladenine 7-glucoside (iP7G), isopentenyladenosine 5-monophosphate (iPMP), *trans*-zeatin (tZ), *trans*-zeatin riboside (tZR), *cis*-zeatin (cZ), *cis*-zeatin riboside (cZR), *trans*-zeatin glucoside (tZ9G), dihydrozeatin (dhZ), isopentenyladenine 7-glucoside (iP7G) (all from OlChemIm, Olomouc, Czech Republic). All substrates were dissolved in DMSO with final concentration of 5% in the reaction mixture.

For determination of specific activities, protein content in the samples was assayed according to the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. An HP Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) was used for absorbance measurement.

*Generation of GFP translational fusions*

A Modular Binary Construct System (Christopher Taylor Lab, Donald Danforth Plant Science Center, St. Louis, MO, USA) was used for *GFP* gene fusions, binary vectors construction and subsequent transformation and overexpression of the *GFP* tagged *ZmCKX1* and *ZmCKX10* genes in tomato hairy roots under a super ubiquitin (SU) promoter. The *ZmCKX10* ORF in pDrive was PCR-amplified using following primers: nsBam_f 5′-CG**GGATCC***ATGATGCTCGCGTACA*-3′, containing *Bam*HI restriction site compatible with the *Bam*HI site in *GFP* prepro region of the AKK1436 vector (bold), and nsBam_r 5′-CG**GGATCC***A**C**ACGGCGACG*-3′ containing *Bam*HI restriction site (bold) and the 3′-untranslated *ZmCKX10* cDNA (italics) with a modification at stop codon region (underlined). By omitting the CT of the stop codon of *ZmCKX10* and subsequent *Bam*HI restriction of the PCR product and AKK1436, an in-frame *ZmCKX10-GFP* fusion was produced. Similarly, an in-frame fusion of *ZmCKX1-GFP* was produced as follows. The *ZmCKX1* (GenBank accession no. NM_001112121) ORF present in the binary vector pROM30 (Bilyeu, unpublished) was PCR-amplified using the primers: ZmBgl_f 5′- GA**AGATCT***ATGGCGGTGGTTTATTACCTGC*-3′ containing *Bgl*II site (bold) and ZmBgl_r 5′-GA**AGATCT**C*G**T**TGAAGATGTCCTGGCCG*-3′. Digesting the amplified product by *Bgl*II, a GATC overhang was created and subsequently ligated with the same overhang of *Bam*HI-digested AKK1436. PCR conditions were used as described in section “Isolation of the *ZmCKX10* gene”. To incorporate the GFP fusions into the new binary vector, a cloning cassette of AKK1436 containing the appropriate GFP-fused gene was cut out by *Pac*I digestion and ligated into *Pac*I digested binary vector AKK1472B. A positive control for overexpression of sole GFP was generated by ligation of the AKK1436 cloning cassette directly into the AKK1472B binary vector. The genes were fused with GFP after a short linker sequence between the genes and GFP (for details see Supplementary Fig. S1 available at *JXB* online).

*Tomato hairy root transformation and cultivation conditions*

Root transformation was performed following the published protocol (Collier *et al.,* 2005). The binary constructs containing SU:*ZmCKX10-GFP*, SU:*ZmCKX1-GFP,* and SU:*GFP* were introduced into *Agrobacterium rhizogenes* strain 15834 (kindly provided by Christopher Taylor, Donald Danforth Plant Science Center, St. Louis, MO, USA). Bacterial cells were grown in MGL media (0.25% yeast extract, 0.5% tryptone, 0.5% sodium chloride, 0.1% L-glutamic acid, 0.5% D-manitol, 0.026% potassium hydrogen phosphate, 0.01% magnesium sulfate hemihydrate, 1 x $10^{-5}$% d-biotin, pH 7.0) supplied with kanamycin (100 mg $l^{-1}$, Sigma-Aldrich). Third day cultures were transferred to 7 mL of fresh media supplied with antibiotics and continuously cultivated for an additional 6 hours. The cultures were subsequently centrifuged for 15 min at 5000 rpm. Bacterial pellets were washed five times with MS medium (Murashige and Skoog basal liquid medium; Sigma-Aldrich) pH 5.8 and resuspended in 10 mL of the medium. The cultures were stimulated by adding acetosyringone to a final concentration of 375 μM.

Tomato seedlings (*Solanum lycopersicum* L.*,* Peto 343) were grown in a growth chamber at 24°C, 12 hour photoperiod (illuminance 1000-1500 lx), for 6-8 days on MS medium at pH 5.8 solidified with 0.8% agar (extra pure; Merck, Darmstadt, Germany). Explants were prepared by cutting cotyledons from the seedlings immediately after their appearance from the seed coat. The cotyledons were wounded by scalpel blade and maintained adaxial leaf surface upwards on D1 agar which consists of MS agar supplied with 100 mM *trans*-zeatin (OlChemIm) for two days. On the second day, the explants were saturated with transformed *A. rhizogenes* 15834 and consequently cultivated on Gamborg's B-5 medium (Sigma-Aldrich) solidified with 1% agar (extra pure, Merck) pH 5.7 for 10-20 days. The regenerated root system represented a composite of transformed and wild-type (WT) tissues. The status of individual regenerated roots was evaluated by assessing GFP fluorescence with a SMZ800 Stereoscopic Microscope (Nikon, Kawasaki, Japan). Individual transgenic roots expressing GFP were excised and placed on 7.0 cm filter paper overlaid on Gamborg's B-5 medium agar pH 5.7 that was amended with 150 mg $l^{-1}$ Timentin (Duchefa) and 2 mg $l^{-1}$ bialaphos (Wako Chemicals GmBH, Neuss, Germany). Cultures were wrapped with Medipor porose adhesive plaster with hydrophobic protected surface (Mediplast, Jilemnice, Czech Republic) and incubated at 26°C in dark to induce root elongation and branching.

*Subcellular localization of GFP-fused proteins*

The roots were excised from their taproot and embedded in 4% low-melting agarose and sectioned into slices (50-100 μm thick) using a VT 1000 vibratom (Leica Microsystems, Wetzlar, Germany). The root sections were mounted in 100 mM phosphate buffered saline (PBS) pH 6.5 prior to observation. For confocal microscopy, a laser scanning inverted microscope IX-81 FluoView 1000

fitted with image processing LCS Imaris 5.0.3 software and a 488-nm argon laser was used (Olympus, Tokyo, Japan). GFP emission of scanned root sections was recorded using a 505-525nm band-pass filter. Prior to the experiment, the proper intensity of lasers was adjusted using control wild-type (WT) tomato hairy roots (induced with WT *A. rhizogenes* 15834).

*Protoplasts*

Protoplasts were prepared using cell wall digestion of tomato hairy root cells. Segments of tomato hairy roots (1mm) were treated by 2% cellulysin (Sigma-Aldrich) and 1% pectinase (Sigma-Aldrich) in 100 mM PBS pH 6.5. The release of protoplasts was observed within 1 h after onset of digestion.

*Analysis of transgenic roots*

To confirm the presence of the transgenes in overexpressing tissue, total RNA was extracted from tomato hairy roots using the RNeasy Plant Mini Kit (Qiagen) and processed in RT-PCR as described above. Transgenes were detected using primer pairs specifically amplifying a unique fragment of *GFP* or tomato actin genes (GFP_f 5´-GAATTAGATGGTGATGTTAATGG-3´ and GFP_rev 5´-CCATGCCATGTGTAATCCC-3´ for *GFP*, TA_fw 5´-CCTTCCAGCAGGTTTGCATT-3´ and TA_rev 5´-GATAGACACCCAAAATAACAGCAAAG-3´ for tomato actin).

CKX activity was quantitated to determine if the overexpressed ZmCKX10-GFP and ZmCKX1-GFP influenced the level of CKX activity of the transgenic roots. Isolation of CKX from transgenic roots was performed following the published protocol (Galuszka *et al.,* 2007). Tomato hairy roots were cut to pieces, powdered in liquid nitrogen using a hand mortar, and extracted with 2-fold excess (v/w) of the extraction buffer containing 0.2 M Tris/HCl pH 8.0, 0.3% Triton X-100, and 1 mM PMSF. Cell debris was removed by centrifugation at 19 500 x *g* for 10 min. The supernatant was used immediately for CKX activity measurement (see CKX activity assay).

*Phenotype determination*

A phenotype of the tomato hairy roots overexpressing GFP fused genes was evaluated by comparing visual appearance (morphologic changes examination) and dry weight of the roots overexpressing SU:*ZmCKX1-GFP*, SU:*ZmCKX10-GFP* and SU:*GFP*. A SMZ800 Stereoscopic Microscope (Nikon) was used for determination of morphologic changes of the roots. To measure dry weight of the roots, 21-day old root tissue produced by the initial root segment was powdered in liquid nitrogen prior to measurement.

## Results

*The sequence of ZmCKX10 gene*

The full length cDNA of *ZmCKX10* (GenBank Accession no. FJ269181) was obtained from RNA of *Z. mays* cv. Cellux 225 kernels using RT-PCR. The primers used for the gene amplification were designed according to sequence information obtained from a survey of the available maize genome database (The Maize Genome Sequencing Project; www.maizesequence.org); a genome region containing the predicted ORF for the *ZmCKX10* gene was localized on the BAC clone AC197220.4 (formerly AZM5_85766). An affiliation of the gene locus to one of the maize chromosomes has not been elucidated yet. Since maize genes are rich in GC content, amplification in the presence of 1.3% DMSO and 1.3% betaine (Henke *et al.*, 1997) appeared to be crucial. The predicted ORF for the *ZmCKX10* gene encodes a putative protein consisting of 504 amino acids with a predicted molecular mass of 53.8 kDa and an isoelectric point of 6.03 (http://expasy.org/tools/). A FAD binding motive common among all studied CKX enzymes was found to be present in the sequence. Database analysis using iPSORT (Bannai *et al.*, 2002; http://hc.ims.u-tokyo.ac.jp/iPSORT/) and TargetP (Emanuelsson *et al.*, 2000), revealed neither N-terminal pre-sequence for any signal peptide nor a nuclear localization sequence. Seventeen clones were selected and sequenced. Alignment of sequences of selected clones and predicted sequence of *ZmCKX10* revealed a discrepancy in the length of the second exon that was spliced on an alternative splicing site among all the clones in comparison to the predicted sequence. Furthermore, other variations among the clones were present. A Clustal W (Thompson *et al.*, 1994) alignment compares differences among the clones and predicted *ZmCKX10* translated sequences (Fig. 1). As it was not clear which sequence encodes a functional enzyme, all three variants were selected for further analysis. All three selected clones contained an extension of the second exon, but clone 1 excluded three residues in this region. Further, the clone 1 sequence excluded an additional three nucleotides resulting in the deletion of alanine A34-; clone 1 also contained two single nucleotide mutations resulting in T25M and N230K changes in the translated sequence. The sequence of clone 2 consisted of the full-length variant of the extended second exon and contained two single nucleotide variations resulting in I126V and N230L mutations. The two mutations of clone 2 were repaired using site-directed mutagenesis to produce clone 3, which differs only in the presence of the extended second exon sequence in comparison to the original *ZmCKX10* sequence predicted from the database. Recombinant proteins of all three clones were prepared to investigate which sequence encoded a functional protein.

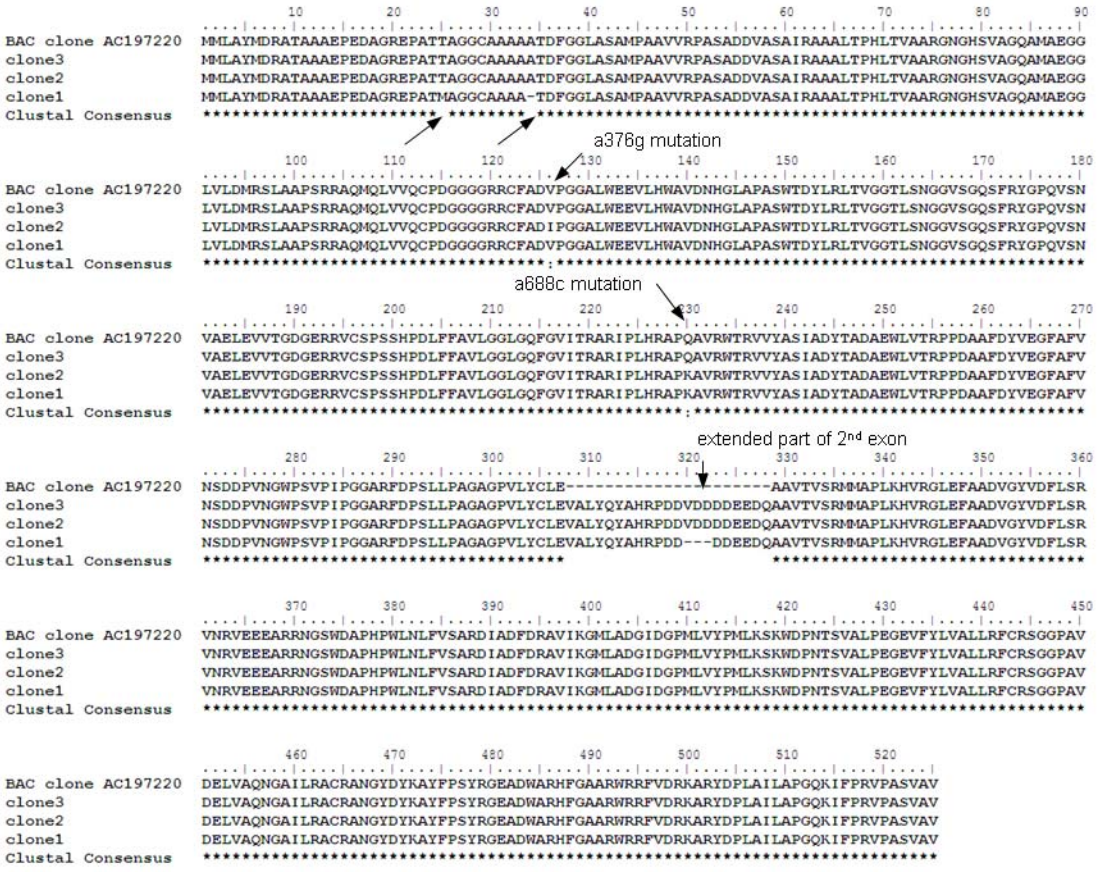


**Fig. 1.** ClustalW alignment of translated sequences of the predicted ZmCKX10 (part of the BAC clone AC197220.4) and the sequences of isolated ZmCKX10 clones. Identical and similar amino acids are marked with asterisk and colon, respectively. Arrows show positions where the sequences differ in comparison with the predicted ZmCKX10 sequence.

*Organ-specific expression of two differentially targeted ZmCKX isoforms*

Real-time RT-PCR was used to determine expression profiles of ZmCKX1 and ZmCKX10. The transcript level of both studied *CKX* genes was determined in different maize organs and developmental stages. Since the transcript abundance of cytokinin metabolizing genes is very low in general, a real-time RT-PCR method was used. The transcripts of both genes were detected in all samples tested; however, the accumulation of *ZmCKX10* transcripts was generally higher than that of *ZmCKX1,* with the exception of the embryo and endosperm (Fig. 2). The expression of *ZmCKX1* was almost undetectable in whole seedlings and in young leaves. The levels of *ZmCKX10* transcripts were fairly similar in the vegetative organs. The highest levels of *ZmCKX10* transcripts were in tassels and silks. Predominant expression of *ZmCKX1* was in developing kernel and reproductive organs, while endosperm was determined as the only tissue where ZmCKX10 was significantly less abundant.

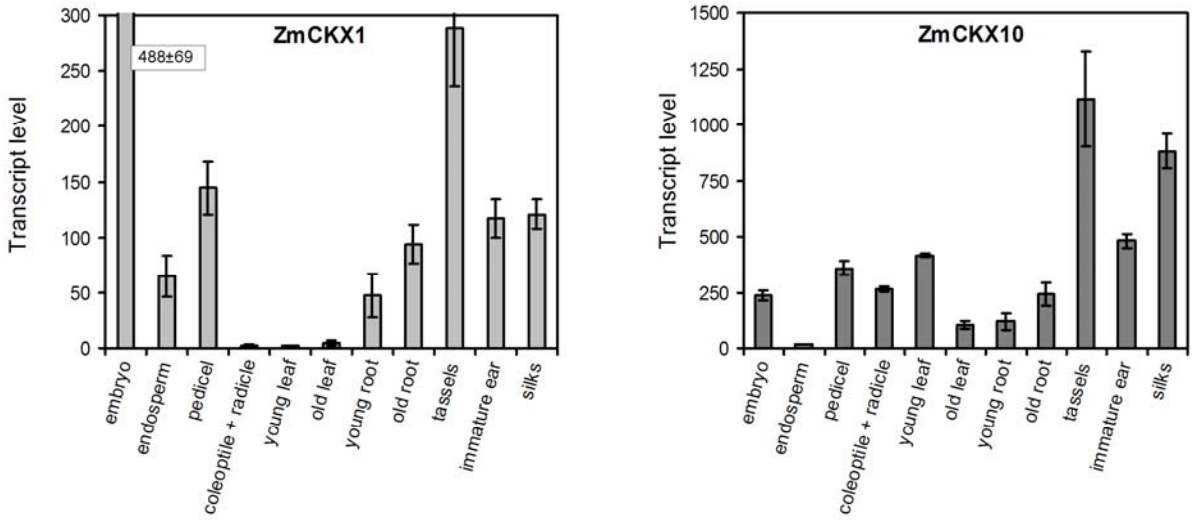


**Fig. 2.** Spatial and temporal expression profile of *ZmCKX1* and *ZmCKX10* genes. The transcript level determined by real-time RT PCR is given as the copy number of particular gene per 1 ng of total RNA isolate from an embryo, which was used as a calibrator for ΔΔCt relative quantification methods corrected by an efficiency factor. The mean values of at least four replicates with ±SD are shown. Transcript levels were normalized with respect to 18S small subunit RNA gene and amplification efficiency obtained from standard curves. For experimental details, see Material and methods.

*Production of recombinant ZmCKX10*

Three different versions of the *ZmCKX10* gene were expressed recombinantly in *Pichia pastoris.* Since the gene lacks a signal peptide, the expression was driven to the cytosol of the yeast host cell, where soluble ZmCKX10 was found. The presence of functional ZmCKX10 protein was detected using a CKX activity assay (Galuszka *et al.,* 2007, Frébort *et al.,* 2002). All the three versions of the gene encoded active proteins as demonstrated by CKX activity assay. Furthermore, there was no difference in the level of CKX activity among the three variants; the specific activity was ~ 8.5 pkat $mg^{-1}$ of extracted intracellular proteins for all three clones when measured with iP as the substrate and DCPIP as the electron acceptor at pH 6.5, or iPR in the presence of $Q_0$ at pH 5.5. The recombinant protein encoded by clone 3 (GenBank Accession No. FJ269181) was further purified by chromatography and characterized as a 56.3 kDa, 525 amino acid protein by mass spectrometry (see Supplementary Fig. S2 at *JXD* online).

*Comparison of ZmCKX10 and ZmCKX1*

Because of the potential for functional divergence between ZmCKX1 and ZmCKX10, both enzymes were compared for their electron acceptor preference and substrate specificity. To determine the electron acceptor preference, the specific CKX activity of the enzymes was measured with iP as a substrate and either DCPIP, ferricyanide, or $Q_0$ as the electron acceptor. A reaction without any electron acceptors (in the presence of oxygen) was set up to determine the basal level of the enzyme activity (Galuszka *et al*., 2001; Frébort *et al*., 2002; Frébortová *et al*., 2004). The most preferable electron acceptor of ZmCKX1 was DCPIP, enhancing its activity 390-fold, while ZmCKX10 activity was only modestly enhanced by ferricyanide, DCPIP, and $Q_0$ (Table 1). Thus, while the activity of secreted ZmCKX1 is dramatically increased in the presence of an electron acceptor, the *in vitro* effect of tested electron acceptors on cytosolic ZmCKX10 activity seems markedly less significant.

**Table 1.** Enhancement of ZmCKX1and ZmCKX10 enzyme activity by artificial electron acceptors

*Pichia pastoris* recombinant proteins ZmCKX10 and ZmCKX1 were compared in terms of their specific CKX activities in the presence of different electron acceptors at pH 6.5 in 200 mM McIlvaine buffer with 50 μM iP as a substrate. As electron acceptors, 100 μM DCPIP, 250 μM $Q_0$ and 250 μM ferricyanide were used, respectively. The values show specific CKX activities and their ratios to the activity obtained without externally added electron acceptor (with $O_2$). The values shown are means ±SD of five independent experiments, a minimum of three samples for each experiment.

| **Electron acceptor** | **ZmCKX1** | | **ZmCKX10** | |
|---|---|---|---|---|
| | **Specific activity (nkat mg$^{-1}$)** | **Ratio to $O_2$** | **Specific activity (nkat mg$^{-1}$)** | **Ratio to $O_2$** |
| **None ($O_2$)** | 5.97±0.27 | 1.0 | 1.10±0.18 | 1.0 |
| **DCPIP** | 2328.1±25.7 | 390.0±4.31 | 8.25±0.63 | 7.5±0.57 |
| **$Q_0$** | 1623.8±43.3 | 272.0±7.26 | 4.07±0.36 | 3.7±0.33 |
| **Ferricyanide** | 284.8±6.74 | 47.7±1.13 | 9.35±0.56 | 8.5±0.51 |

To compare the substrate specificity of both enzymes, a continual method for CKX activity determination was used (Laskey *et al.*, 2003). Different substrates were tested in separate reactions and the specific activities were assessed and compared (Fig. 3). The most preferable substrates of ZmCKX1 were *trans*-zeatin, isopentenyladenine and isopentenyladenosine (as previously reported; Frébortová *et al*., 2004); in contrast, ZmCKX10 efficiently degraded *cis*-zeatin and cytokinin 9-glucosides in addition to *trans*-zeatin, isopentenyladenine and isopentenyladenosine.

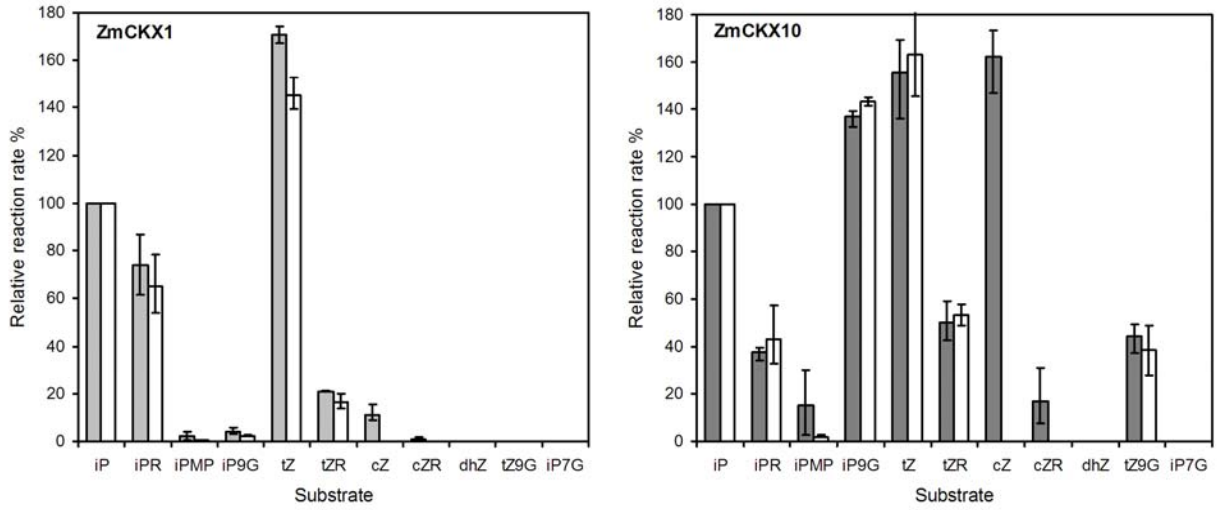


**Fig. 3.** Comparison of the substrate specificity of ZmCKX1 and ZmCKX10 expressed as relative reaction rates towards iP (specific activity measured with iP is given as 100%). The values were estimated by continuous (filled bars) and discontinuous (open bars) CKX activity assay. All analysed substrates were used in 50 μM concentrations at pH 6.5 in 200 mM McIlvaine buffer with 100 μM DCPIP as the electron acceptor. For the continuous assay, decolorization of DCPIP was monitored at 600 nm (Laskey *et al.*, 2003), while for the discontinuous assay, the amount of the aldehyde product was assayed with 4-aminophenol (Frébort *et al.* 2002). The values for cZ and cZR cannot be determined by the latter assay as their CKX reaction product does not react with 4-aminophenol. The values shown are means ±SD of five independent experiments, with a minimum of four samples for each experiment.

*Tomato hairy root transformation and subcellular localization of ZmCKX1 and ZmCKX10*

Whereas ZmCKX1 was confirmed to be localized to the apoplast (Morris *et al.*, 1999; Galuszka *et al.*, 2005), ZmCKX10 was predicted to lack any translocation signal and therefore should function in the cytosol. To confirm the predicted localization of ZmCKX10 and compare it with ZmCKX1, the genes were fused on their C-termini with *GFP*. The fused genes were constitutively expressed in *A. rhizogenes*-induced tomato hairy roots. The transgenic root samples were analysed for fluorescence patterns using confocal microscopy. As shown in Figure 4B, the diffuse fluorescence pattern attributable to transiently expressed ZmCKX10-GFP was similar to the fluorescence of transiently expressed GFP where a characteristic pattern can be found throughout the cytosol, accumulating along the plasma membrane and in nuclei (Grebenok *et al.*, 1997a, b; Köhler *et al.*, 1997b; Haseloff and Siemering, 1998; Fig. 4A). It is known that GFP can enter the nuclei through nuclear pores, but large fusion proteins should not enter the nuclei. However, an experiment with a GFP dimer (55 kDa) and trimer (83 kDa) has shown that even though the fact that fluorescence remains primarily cytosolic, there are still remains of fluorescence accumulating around the nuclei (Arnim *et al.*, 1998). Accordingly, the 82 kDa ZmCKX10-GFP fusion protein containing no translocation signal showed cytosolic location. Whereas ZmCKX10-GFP was localized in cytosol, the ZmCKX1-GFP fluorescence pattern was visible only in the perimeters of the cells, confirming its localization in the apoplasts (Fig. 4C). To determine whether ZmCKX1 is actually associated with the plasma membrane, the cells were treated by cellulases to release protoplasts. Cellulase treatment led to the disappearance of the GFP fluorescence, indicating there are no interactions of ZmCKX1 with the plasma membrane (Fig. 4F). Protoplasts released from SU:*GFP* (Fig. 4D) and SU:*ZmCKX10-GFP* (Fig. 4E) were analysed as controls, and confirmed their intra-cellular fluorescence signals. The presence of the transgenes was confirmed using RT-PCR (see Supplementary Fig. S3 at *JXB* online).

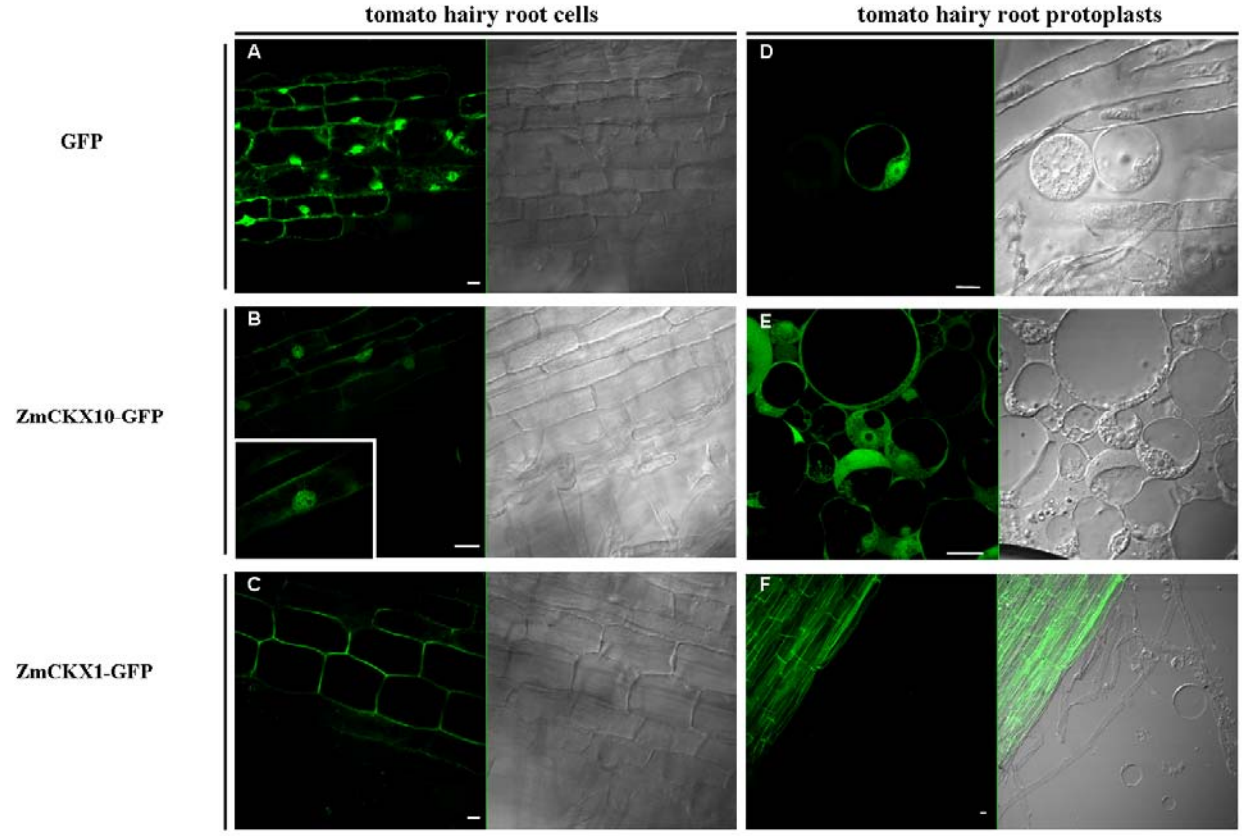


**Fig. 4.** Subcellular localization of GFP fusion proteins. Green channel and transmission light images were captured by confocal microscopy. **(A)** A control root, overexpressing SU:*GFP*, shows typical cytosolic localization of GFP with fluorescence along plasma membrane in the cytosol and in the nuclei of the cells. **(B)** The root cells overexpressing SU:*ZmCKX10-GFP* containing no N-terminal signal peptide resemble the sole GFP fluorescence signal indicating the cytosolic localization of ZmCKX10. The inset in **(B)** shows detail of the root cell with signal visible throughout the cytosol and in nuclei. **(C)** The fluorescence signal of ZmCKX1-GFP indicates its secretion out of the cell to the protoplast region. Whereas protoplasts released from SU:*GFP* **(D)** and SU:*ZmCKX10-GFP* **(E)** retain the fluorescence in the cytosol, the protoplast released from SU:*ZmCKX1-GFP*, visible on the transmission and green channel merged image **(F),** possess no more fluorescence, suggesting there are no interactions of ZmCKX1 with the plasma membrane. Scale bars indicate 10 μm.

*CKX activity and phenotype determination of ZmCKX10-GFP and ZmCKX1-GFP overexpressing roots*

Cell extracts of transgenic tomato hairy roots were tested for CKX activity. The cell extracts were analysed with iP and DCPIP or with iP9G and $Q_0$, as the substrate and electron acceptor, respectively. Tomato hairy roots (WT; induced by wild-type *Agrobacterium rhizogenes* 15834) and tomato hairy roots overexpressing SU:*GFP* cell extracts were used as controls. The specific CKX activity of transgenic tissue was determined and expressed as a ratio of the CKX activity value to the activity of the control roots. While the CKX activity of the roots overexpressing ZmCKX10-GFP with iP9G was found to be 1.68-fold higher than in the controls, the ZmCKX1-GFP enzyme activity measured with iP was three orders of magnitude (1564-fold) higher than the controls (Table 2).

**Table 2.** CKX activity assay of transgenic tomato hairy roots

Cell extracts of tomato hairy roots overexpressing GFP, ZmCKX1-GFP and ZmCKX10-GFP or WT roots were analysed for CKX activity in the presence of 250 μM electron acceptor and 100 μM substrate in 200 mM McIlvaine buffer pH 6.5 or 5.5, respectively. The values shown are means ±SD of six independent experiments with a minimum of three cell extract samples for each experiment. The ratio of the mean of CKX activity of WT and GFP overexpressing roots (both are equal in CKX activity level) was considered to be 1.0 and was set up to express difference in CKX activity among analysed transgenic tissues.

| **Overexpressed transgene** | **Specific activity[*] (pkat mg$^{-1}$)** | **Ratio to WT** | **Specific activity[†] (pkat mg$^{-1}$)** | **Ratio to WT** |
|---|---|---|---|---|
| **WT, GFP** | 5.57±0.31 | 1.0 | 5.07±0.38 | 1.0 |
| **ZmCKX1-GFP** | 8709[‡] | 1564[‡] | 5.17±0.61 | 1.0 |
| **ZmCKX10-GFP** | 5.61±0.39 | 1.0 | 8.44±0.16 | 1.68 |

[*] Measured with DCPIP as the electron acceptor and iP as the substrate at pH 6.5.

[†] Measured with $Q_0$ as the electron acceptor and iP9G as the substrate at pH 5.5.

[‡] The values vary from 2 373 pkat mg$^{-1}$ to 8 709 pkat mg$^{-1}$ depending on the fluorescence intensity of the transgenic roots (probably due to varying efficiency of translation and correct folding).

To find out whether the transgenic roots displayed any phenotypic modifications, the visual appearances of the roots were assessed for morphologic modifications. No morphologic changes among transgenic roots were observed; the only difference was in the rate of tissue production. Comparing the different transgene overexpressing roots, the roots overexpressing ZmCKX1-GFP showed faster root formation resulting in higher tissue production when compared to the control WT roots or roots overexpressing the sole GFP (see Supplementary Fig. S4 at *JXB* online). In contrast to ZmCKX1-GFP, ZmCKX10-GFP overexpressing roots showed retarded root formation resulting in reduced tissue production. To determine the difference in root mass production among transgenic roots, a dry weight of 21-day old roots was specified and compared (Fig. 5). The results confirm a slight enhancement of root production for ZmCKX1-GFP roots compared to the control and ZmCKX10-GFP roots.

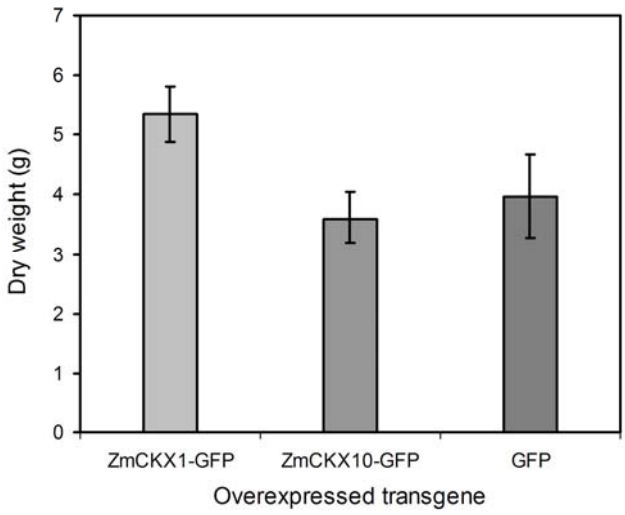


**Figure 5.** Comparison of the rate of tissue production

The 21-d-old transgenic root tissue produced by the initial root was ground to a powder in liquid nitrogen, while the total weight of the tissue was measured. The values shown are means ±SD from three independent experiments with a minimum of eight root samples for each experiment. The roots overexpressing ZmCKX1-GFP corresponds to 134.5% and ZmCKX10-GFP to 90.3%, when the dry weight of GFP overexpressing roots is given as 100%.

## Discussion

The first cloned member of the maize CKX family, *ZmCKX1*, was extensively biochemically characterized, predicted to contain a secretion signal peptide, and later confirmed to be localized to the apoplast region (Morris *et al.*, 1999; Galuszka *et al.*, 2005). Screening the Maize Genome Sequencing Project databases revealed at least 13 entries showing significant sequence identity to the annotated CKX. Some of the maize CKX sequences found appear to be the result of relatively recent duplications. All but one of these sequences were predicted to have a signal peptide. Considering diverse environments of the cellular compartments, specific roles of the differently targeted CKX enzymes in cytokinin homeostasis in plants may be suggested. Therefore, it was particularly intriguing to explore the function of the one maize CKX that lacks a signal peptide. In this work, the non-secreted *CKX* gene was isolated from maize; the biochemical properties of its protein were characterized and found to be distinct from those of the secreted ZmCKX1. Furthermore, the subcellular localization was defined and contrasted with that of the secreted ZmCKX1.

The gene coding for ZmCKX10 was successfully cloned as described above. According to obtained data, the cDNA of the *ZmCKX10* gene (GenBank Accession No. FJ269181) consisted of 1578 bp. A discrepancy of 63 bp with the predicted ORF was found among all analysed clones that was caused by an extension of the second exon in comparison with the predicted 1515 bp *ZmCKX10* sequence (BAC clone AC197220.4). Figure 6 illustrates the region where the discrepancy of 63 bp is located. An expressed sequence tag (EST) analysis was carried out using BLAST at the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to elucidate the sequence variation in the extended region of the second exon. Since only four EST sequences were found to match the region, obtained information is limited. The EST sequence GenBank Accession No. EC875373 contains the 63 bp extension, whereas other three matching EST sequences (EE013728, CO453006 and FL282551) preserve the full length of 674 bp. Thus these annotated ESTs might have arisen from genomic DNA contamination or code for an alternatively spliced protein different from CKX. Since special conditions (described in Materials and methods) for *ZmCKX10* amplification were used, frequent point mutations were encountered varying among the analysed clones. However, there were three different patterns identified repeating throughout the sequences of the clones. All the three variants of the *ZmCKX10* were overexpressed in *Pichia pastoris* and proved to code for functional proteins. The basis for these variants is not known, although it is possible they resulted from the pedigree of the maize line used as the source of RNA. It is concluded that there is a high variability in the maize gene transcript for non-secreted CKX.

maizesequence, BAC clone AC197220.4:1-101899 selected region 39353 to 40555 (-)

**CGGTGGACGCGCGTGGTGTACGCGAGCATCGCGGACTACACGGCGGACGCGGAGT**
**GGCTGGTGACGCGGCCCCCCGACGCGGCGTTCGACTACGTGGAGGGCTTCGCGTT**
**CGTGAACAGCGACGACCCCGTGAACGGCTGGCCGTCCGTGCCCATCCCCGGCGGC**
**GCCCGCTTCGACCCGTCCCTCCTCCCCGCCGGCGCCGGCCCCGTCCTCTACTGCC**
**TGGAGG**TGGCCCTGTACCAGTACGCGCACCGGCCCGACGACGTCGACGACGACGA
TGAGGAGGACC**AGG**TAGGTAGCAGTAATTGCCAACCTCTCCCCCCGCTGAGACTT
GGCGCATTCCCGTACTTGACCCCCTCGCCCGCTCTGGCGTGTACTTTTCCGCGGG
CAGGGCATGTCTGACTCGCCTCGTCGTGTATCTCCCGCTGGATTCGGTGACGGGG
GGGCTGCGTCCTGCCAAACCAAACCACCCTAGACTAGACAGACCCCCAGGGGCAG
GGGTCGCGCCATTGGCCGCACGCGGGGACCGGCGCCAGTGAGTGCGCCGCGCCGC
ACGGCCGCGCCCCGATCTCGCTCGCTCGCTCGCTGGTGATCGAATCGGCGCGTAC
AATGCGGCATGGCCCCGAGCCCCACACCCGCAGTGGCCGTGACGCGATTGCGCTG
CCTCCGGTCCGGCCCATGACCCAGCGGATCGCGTCGCGTCTTTTGGCAACGCCCG
CGTCATCATATCGCGCTCTTTGTCGTCCCCACGGAGCACAGCGCAGCGCAGCGCA
GCGCAGCCAACCTTTTCTCCGCCACGCACGCTT**CGGCGGCATTCATTATTTGGAT**
**TTTGTTCCTACCGGTCGATCCGCGTCCGTCCGTGCACTGCAGGCGCTACCGTCAT**
**GCTGACCAACCCATTGCCATTGGTTTTGTTTCTTCTCTCTCTCTCTCGCTCTCGT**
**TGGTTATGGTTCGTGCGTGCCTGCAGGCGGCGGTGACCGTGAGCCGGATGATGGC**
**GCCGCTCAAGCACGTGCGGGGCCTGGAGTTCGCGGCGGACGTCGGGTACGTGGAC**
**TTCCTGTCCCGCGTGAACCGGGTGGAGGAGGAGGCCCGGCGCAACGGCAGCTGGG**
**ACGCGCCGCACCCGTGGCTCAACCTCTTCGTCTCCGCGCGCGACATCGCCGACTT**
**CGACCGCGCCGTCATCAAGGGCATGCTCGCCGACGGCATCGACGGGCCCATGCTC**
**GTCTACCCTATGCTCAAGAGCAAGTG**

**Fig. 6.** Genomic region section of predicted *ZmCKX10* BAC clone AC197220.4:1-101899 39353 to 40555 (-). The proposed second exon (bold) ends at predicted splicing site (AGG) followed by the intron (normal), where the preferred splicing site (AGG) is indicated (bold). The following third exon is indicated (bold).

The recombinant ZmCKX10 protein was purified and compared in terms of its electron acceptor preference and substrate specificity with ZmCKX1. In contrast to ZmCKX1, whose most preferred substrate is *trans*-zeatin, ZmCKX10 preferentially degraded *cis*-zeatin, when determined by continuous method of CKX activity assay. High levels of *cis*-zeatin derivatives are primarily found in vegetative organs of maize where *trans*-zeatin is nearly undetectable. On the other hand, *trans*-zeatin and its derivatives in contrast to *cis*-isomers are massively distributed throughout developing kernels (Veach *et al*., 2003). Expression of ZmCKX1 is highest in developing kernels where ZmCKX10 transcripts have relatively lowest abundance. In contrast, in vegetative tissues, levels of ZmCKX10 are significantly higher than those of ZmCKX1. Thus, it can be generalized that particular CKX isoform is localized in tissue or organs where its preferable substrate is also allocated. The distribution of both zeatin isomers was equal in immature ears, where expression of both *CKX* genes was strong, as well as in tassels and silks.

Divergence of both isoenzymes was apparent in their preference for 9-glucosylated substrates. While ZmCKX10 degrades iP9G and tZ9G very effectively, the same substrates are almost unaffected by the activity of ZmCKX1. This is in agreement with the substrate specificity of *Arabidopsis thaliana* cytosolic AtCKX7 and apoplastic CKXs demonstrated previously (Galuszka *et al.*, 2007). Turnover

rates of both enzymes were enhanced by all the tested electron acceptors. Whereas the activity of apoplastic ZmCKX1 was enhanced by DCPIP the most (390-fold), none of the tested electron acceptors showed such a significant enhancement of the turnover rates for the cytosolic ZmCKX10 enzyme. However, it is notable that the activity of ZmCKX10 was 8.5-fold higher in the presence of ferricyanide and 7.5-fold higher in the presence of DCPIP than without any electron acceptor (in the presence of oxygen). Further biochemical studies concerning the catalytical mechanism should be performed to determine the ability of ZmCKX10 to use other electron acceptors or oxygen and possible mixed mode of action in presence of artificial electron acceptors must be taken into account. Besides, the cytokinin depletion process in the cytosol might not need to be enhanced so significantly in this manner.

GFP fusions of the studied genes and their subsequent overexpression in tomato hairy roots resulted in the confirmation of the ZmCKX1 localization to apoplast and established the predicted localization of ZmCKX10 to the cytosol. The images captured by confocal microscopy show that the fluorescent signal of ZmCKX10-GFP is localized to cytosol and also in nuclei. However, the intensity of fluorescence of the ZmCKX10-GFP 82 kDa protein in nuclei is a remnant when compared with the fluorescent pattern of SU:*GFP* that was used as a positive control. The partial bidirectional diffusion of larger proteins back to the nuclei was described by Arnim (Arnim *et al.*, 1998). Comparing the CKX activity of the roots overexpressing the GFP fusion proteins, an enormous difference was observed. As the CKX activity of the SU:*ZmCKX1-GFP* cell extract was 1564 times higher then that of WT (and SU:*GFP*), no significant changes in the level of CKX activity of SU:*ZmCKX10-GFP* were detected when measured with DCPIP and iP as the substrate. When measured with $Q_0$ and iP9G as a preferred ZmCKX10 substrate, the CKX activity of the cell extract of SU:*ZmCKX10-GFP* was detected 1.68 times higher than that of WT (and SU:*GFP*) roots and was also higher than in roots transformed with SU:*ZmCKX1-GFP*. SU:*ZmCKX1-GFP* overexpressing roots were detected to be no more active than WT and SU:*GFP* under those assay conditions. The conditions of the measurement were chosen based on previous experience with determining CKX activity in cell extracts of plants overexpressing different *Arabidopsis thaliana* CKX genes, where those cell extracts that were not so sensitive to iP as a substrate were much more sensitive to iP9G, especially from the AtCKX7 (non-secreted) overexpressing plant (Galuszka *et al.*, 2007). Phenotypic characterization of the transgenic roots revealed a difference in the rate of root growth. The roots overexpressing SU:*ZmCKX1-GFP* were ascertained to form roots faster with 34.5% more tissue produced when compared to control roots. On the contrary, roots overexpressing *ZmCKX10-GFP* showed an ~10% reduction in root formation. Hence, the root mass accumulation difference between SU:*ZmCKX10-GFP* and SU:*ZmCKX1-GFP* was ~44%. This difference indicates potentially opposing aspects of cytokinin regulation between the cytosol and apoplast impacting the physiology of root growth. Nevertheless, creating a whole plant overexpressing cytosolic CKX would provide more insight into the

physiological implication of compartmentalization of cytokinin degradation. The results reported here suggest that non-targeted ZmCKX10 is a cytosolic enzyme of the maize CKX family that differs in enzymatic properties compared to targeted CKX isoforms. A considerable amount of further work is necessary to determine the nature of this unique cytosolic form of cytokinin dehydrogenases and how this class of enzyme (E.C. 1.5.99.12) is contributing to the complexity of cytokinin homeostatic regulations in plants.

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**Supplementary data**

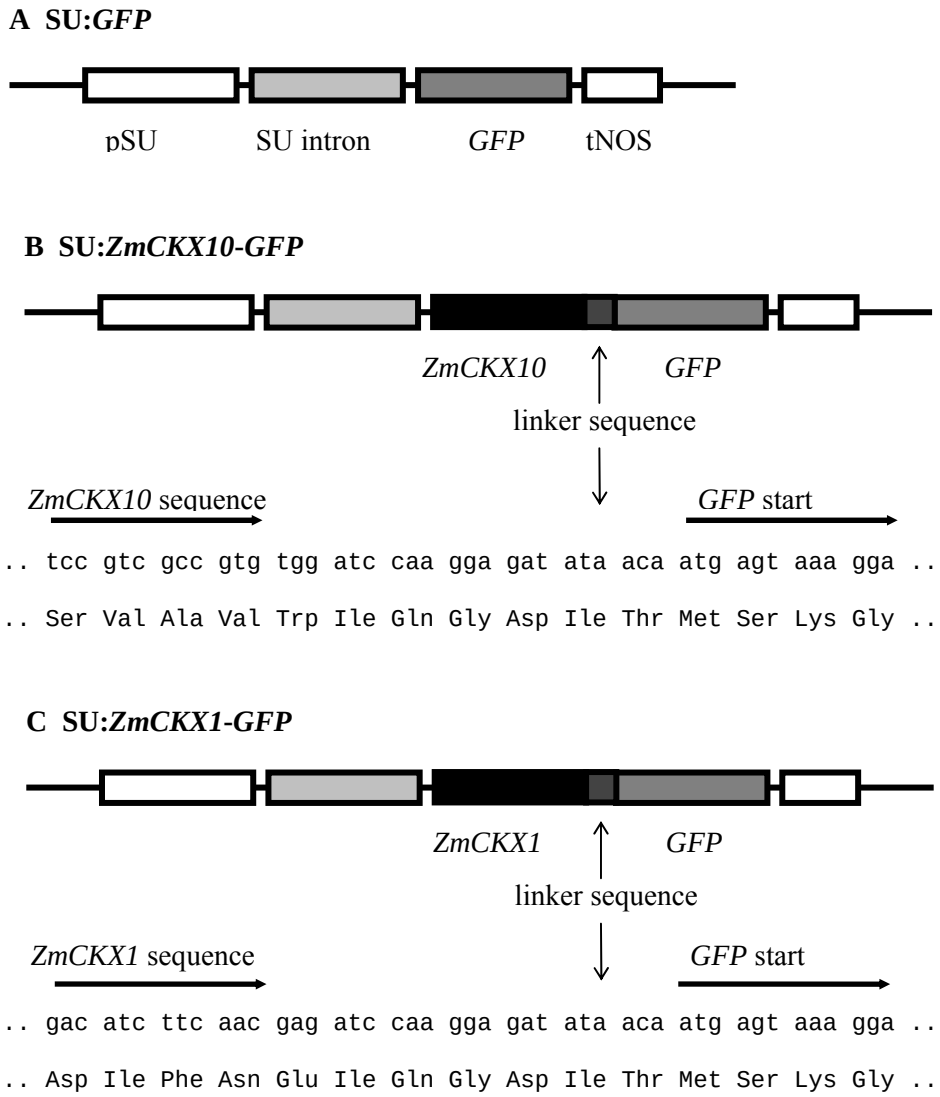


**Fig. S1.** GFP constructs used for tomato hairy root transformation. (A) Control vector, GFP coding sequence under control of the SU promoter, (B) *ZmCKX10* coding sequence missing a STOP codon fused to the 5´-end of *GFP* after linker sequence, (C) *ZmCKX1* coding sequence missing a STOP codon fused to the 5´-end of *GFP* after linker sequence. DNA and amino acid sequences of the fused regions are shown.

Sequence coverage by matched peptides – 43%

MMLAYMDRAT AAAEPEDAGR EPATTAGGCA AAAATDFGGL ASAMPAAVVR PASADDVASA 060
IRAAALTPHL TVAARGNGHS VAGQAMAEGG LVLDMRSLAA PSRRAQMQLV VQCPDGGGGR 120
RCFADVPGGA LWEEVLHWAV DNHGLAPASW TDYLRLTVGG TLSNGGVSGQ SFRYGPQVSN 180
VAELEVVTGD GERRVCSPSS HPDLFFAVLG GLGQFGVITR ARIPLHRAPQ AVRWTRVVYA 240
SIADYTADAE WLVTRPPDAA FDYVEGFAFV NSDDPVNGWP SVPIPGGARF DPSLLPAGAG 300
PVLYCLEVAL YQYAHRPDDV DDDDEEDQAA VTVSRMMAPL KHVRGLEFAA DVGYVDFLSR 360
VNRVEEEARR NGSWDAPHPW LNLFVSARDI ADFDRAVIKG MLADGIDGPM LVYPMLKSKW 420
DPNTSVALPE GEVFYLVALL RFCRSGGPAV DELVAQNGAI LRACRANGYD YKAYFPSYRG 480
EADWARHFGA ARWRRFVDRK ARYDPLAILA PGQKIFPRVP ASVAV

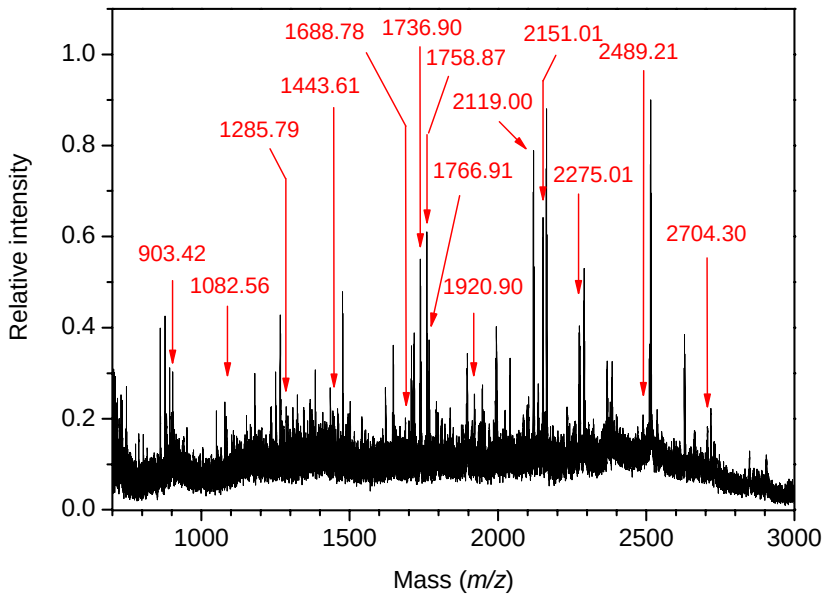


**Fig. S2.** MALDI/TOF mass spectrometry identification of ZmCKX10 recombinant protein. The data show fragmentation of *Zea mays* non-secreted cytokinin dehydrogenase recombinant enzyme, where 43% peptides were matched in the protein sequence. In-gel digestion was done without prior reduction/alkylation and the protein was analysed according to a published protocol (Pospíšilová *et al.*, 2008).

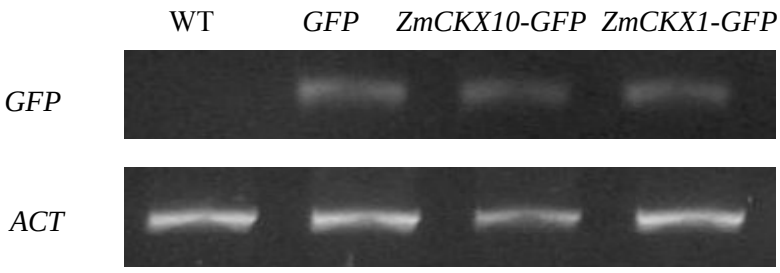


**Fig. S3.** Analysis of transgene presence in transgenic tomato hairy roots. RT-PCR was performed on the cDNA obtained from wild-type (WT), SU:*GFP*, SU:*ZmCKX10-GFP* and SU:*ZmCKX1-GFP* tomato hairy roots. The gene specific primers for tomato actin (*ACT*) and green fluorescent protein (*GFP*) described in Materials and methods were used.

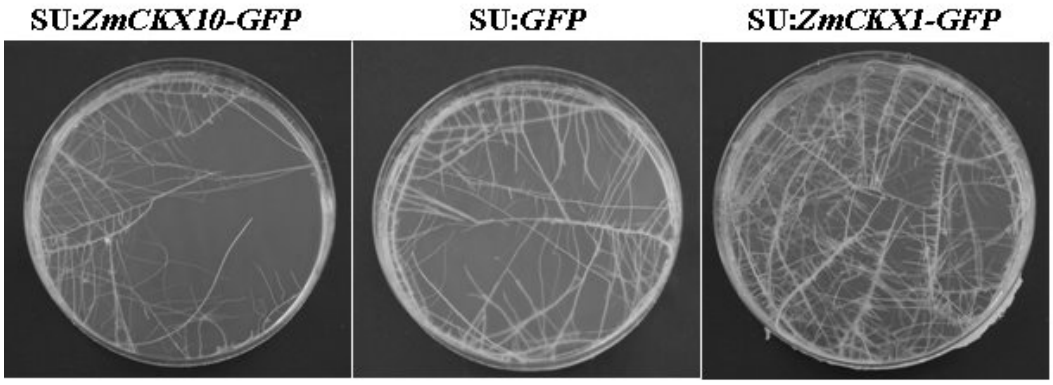


**Fig. S4.** The 21-days old transgenic tomato hairy roots overexpressing SU:*GFP*-fused genes. Based on the measurement of dry weight of transgenic roots, the rate of roots production overexpressing SU:*ZmCKX10-GFP* is slightly lower, whereas the production of the roots overexpressing SU:*ZmCKX1-GFP* is significantly higher, when compared to control roots overexpressing SU:*GFP*.

**Curriculum Vitae**

*Personal Data*


| | |
|---|---|
| First Name: | Mária |
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*Education and study*

| | |
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| 2001 - 2004 | Graduated in Biochemistry (Bc.) in 2004 at the Faculty of Science, Palacký University, Olomouc; Bachelor thesis "Use and purification of the termostable DNA-polymerase expressed in *Escherichia coli*". |
| 2004 - 2006 | Graduated in Biochemistry (M.Sc.) in 2006 at the Faculty of Science, Palacký University, Olomouc; Diploma thesis "Expression systems for production of recombinant proteins". |
| 2006 - 2010 | Ph.D. study program in Biochemistry at the Faculty of Science, Palacký University, Olomouc; theme "Cytokinins as mediators of plant physiological responses" (advisor Petr Galuszka). |
| 2007 - present | Research assistant at the Department of Biochemistry, Faculty of Science, Palacký University, Olomouc. |

*International Scholarship*

| | |
|---|---|
| 2006 | March/April, Free University of Berlin, Germany – Palacký University student internship - focused on "Yeast Two Hybrid System" method. |
| 2006 - 2007 | October – June, University of Missouri, Columbia, USA – Research scholar stay with participation on "Biochemical and structural comparison of two differentially targeted cytokinin oxidase/dehydrogenase enzymes" project. |

*Grants and projects completed*

| | |
|---|---|
| 2009 | FRVS Project No. 1501 (G4) "Plant material transformation by micro projectile DNA transfer and recombinant protein visualization". |

*Achievements*

| | |
|---|---|
| 2008 | Section of Chemistry Award - 1st Prize for The Best Students' Scientific Work of the Department of Biochemistry, Section of Doctoral Thesis<br>Dean's Award – 2nd Place, The Best Scientific Work of The Section of Chemistry, Doctoral Thesis Section |

**List of Author's Publications**

**Šmehilová M.**, Galuszka P., Bilyeu K.D., Jaworek P., Kowalska M., Šebela M., Sedlářová M., English J.T., Frébort I. (2009) Subcellular localization and biochemical comparison of cytosolic and secreted cytokinin dehydrogenase enzymes from maize. J. Exp. Bot. 60 (9), 2701-2712. IF=4.001

Vyroubalová Š., Václavíková K., Turečková V., Novák O., **Šmehilová M**., Hluska T., Ohnoutková L., Frébort I., Galuszka P. (2009) Characterization of new maize genes putatively involved in CK metabolism and their expression during osmotic stress in relation with cytokinin levels. Plant Physiol 151, 433-447. IF=6,110

**Šmehilová M.**, Vyroubalová Š., Szüčová L., Mik V., Spíchal L., Dobrůšková J., Zalabák D. and Galuszka P. (2010) Cytokinin metabolism gene expressions profiles in *Arabidopsis* leaf during senescence: response to kinetin $N^9$-substituted derivatives (Manuscript).

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