PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Department of Biochemistry/Centre of the Region Haná

for Biotechnological and Agricultural Research

Cytokinin dehydrogenase: heterologous expression, isozyme characteristics and analytical application

Ph.D. Thesis

Author:	**Mgr. Ing. Marta Kowalska**
Study program:	P1416 Biochemistry
Supervisor:	Prof. RNDr. Ivo Frébort, CSc., Ph.D.
Date:	30.09.2010

I herby declare that this Ph.D. thesis has been written solely by myself. All the sources quoted in this work are listed in the "Reference" sections. All published results included in this work are approved by co-authors.

September 30, 2010

……..

Acknowledgement

I would like to thank my supervisor Ivo Frébort for his kind patronage, suggestions and critical reading of this work. My thanks belong to the staff of the Department of Biochemistry and the Centre of the Region Haná, especially to Marek Šebela and Petr Galuszka for their help, valuable advices and generous support. I also wish to thank Richard Napier from the University of Warwick for his interest in my work that started our collaboration, for his help with organizing my internship at the University of Warwick and all his indispensable comments on the biosensor project. Last but not least, I thank my family and friends who encouraged and motivated me and all co-authors of Kowalska *et al.* (2010) paper presented in the chapter 2.

Bibliografická identifikace

Jméno a příjmení autora	Mgr. Ing. Marta Kowalska
Název práce	Cytokinin dehydrogenasa: heterologní exprese, charakterizace isoenzymů a analytická aplikace
Typ práce	Disertační
Pracoviště	Katedra biochemie a Centrum regionu Haná pro biotechnologický a zemědělský výzkum
Vedoucí práce	Prof. RNDr. Ivo Frébort, CSc., Ph.D.
Rok obhajoby práce	2010

Abstrakt

Tato práce se zabývá enzymem cytokinin dehydrogenasa z *Arabidopsis thaliana* a je rozdělena do čtyř částí. První kapitola uvádí isoenzymy cytokinin dehydrogenasy, jejich strukturu, lokalizaci, vlastnosti a funkce. Druhá část této práce je zaměřena na purifikaci a charakterizaci nesekretovaných isoenzymů. Vakuolární proteiny AtCKX1 a AtCKX3 byly produkovány v kvasince *Pichia pastoris* a cytosolický enzym AtCKX7 byl získán v expresním systému *E. coli*. V případě AtCKX1 a AtCKX3 byla odhalena přítomnost *N*-terminálního sekvenčně-specifického signálu pro transport do vakuoly. Příslušné rekombinantní proteiny byly purifikovány za účelem biochemické charakterizace. Jejich identita byla potvrzena pomocí MALDI-TOF/MS a následně byly stanoveny jejich substrátové specifity a preference pro elektronové akceptory.

Ve třetí části práce je popsána produkce apoplastického enzymu AtCKX2. Konstitutivní, extracelulární exprese AtCKX2 byla provedena v kvasinkách *Pichia pastoris*. Rekombinantní protein byl označen histidinovou kotvou následovně: na *C*-konci, na *N*-konci a na obou koncích za účelem usnadnění purifikace a byl určen vliv histidinových fúzi na enzymovou aktivitu. Dále byla zavedena exprese ve velkém měřítku, v 15-i litrovém fermentoru, s použitím glycerolu a glukózy, jako zdrojů uhlíku. V poslední kapitole je popsán vývoj cytokininového biosenzoru s využitím rekombinantního enzymu AtCKX2.

Klíčová slova	Cytokinindehydrogenasa; *Pichia pastoris;* Exprese a purifikace proteinů; His-tag; Sekvenčně-specifický signál pro transport do vakuoly; Fermentor; Biosenzor
Počet stran	84
Počet příloh	-
Jazyk	Anglický

Bibliographical identification

Author's name	Mgr. Ing. Marta Kowalska
Title	Cytokinin dehydrogenase: heterologous expression, isozyme characteristics and analytical application
Type of thesis	Ph.D.
Department	Department of Biochemistry and the Centre of the Region Haná for Biotechnological and Agricultural Research
Supervisor	Prof. RNDr. Ivo Frébort, CSc., Ph.D.
The year of presentation	2010

Abstract

This work deals with cytokinin dehydrogenase enzymes from *Arabidopsis thaliana* and consists of four parts. The first chapter is an introduction to cytokinin dehydrogenase enzymes, their structure, localization, properties and functions. The second part of this work is focused on the purification and characterization of non-secreted AtCKX isozymes. Vacuolar proteins AtCKX1 and AtCKX3 were produced in *Pichia pastoris* and cytosolic enzyme AtCKX7 was obtained from *Escherichia coli* expression system. In the case of AtCKX1 and AtCKX3 the presence of *N*-terminal sequence-specific vacuolar sorting signal was revealed. Respective recombinant proteins were purified for their biochemical characterization. With the use of MALDI-TOF/MS the enzymes were identified and subsequently their substrate specificity and electron acceptor preference was determined.

In the third part of the work the production of an apoplastic enzyme AtCKX2 is described. The constitutive extracellular expression of AtCKX2 was carried out in *Pichia pastoris*. Recombinant protein was fused with His-tag domain on its *C*-terminus, *N*-terminus and on both termini in order to facilitate purification and the influence of such fusions on enzyme's activity was determined. Further, large-scale expression in 15 litres fermentor was established with glycerol and glucose as the carbon source. The last chapter describes the development of cytokinin biosensor with the use of recombinant AtCKX2 enzyme.

Keywords	Cytokinin dehydrogenase; *Pichia pastoris;* Protein expression and purification; His-tag; Sequence-specific vacuolar sorting signal; Fermentor; Biosensor
Number of pages	84
Appendices	-
Language	English

Table of contents

Summary

This work deals with cytokinin dehydrogenase enzymes from *Arabidopsis thaliana*. Cytokinins are important signalling molecules in plant cells regulating cell division and differentiation. The levels of cytokinin active forms and their distribution in tissues of many lower and higher plants and likely in some photosynthetic and plant-pathogenic bacteria are controlled by the degradation metabolism. The enzyme playing a key role in cytokinin catabolism is cytokinin dehydrogenase (CKX; EC 1.5.99.12), a flavoprotein that irreversibely cleaves off the N^6-side chain of cytokinins. In different higher plant species CKX proteins are encoded by small gene families with a varying number of members. The *CKX* gene family of *Arabidopsis thaliana* comprises seven members (*AtCKX1-AtCKX7*). AtCKX isoenzymes differ in their biochemical properties, in the regulation of their expression and in the subcellular localization of the gene product. The aim of the work was to obtain active recombinant AtCKX proteins in a heterologous system and to study their biochemical properties.

Pichia pastoris was selected as a suitable host for expression of AtCKX genes as it offers economy, ease of manipulation, the ability to perform complex posttranslational modifications and high expression levels. AtCKX genes without native signal peptide sequences were subcloned into pGAPZα vector and transformed into yeast *Pichia pastoris* X-33. Recombinant proteins were expressed constitutively with secretion into the culture medium. In the case of AtCKX1 and AtCKX3, *N*-terminal sequence-specific vacuolar sorting signal (ssVSS) was determined. Only after deleting this signal, active plant vacuolar cytokinin dehydrogenases were obtained in *Pichia pastoris*. In order to facilitate purification, recombinant proteins were fused with His-tag domain. After optimization of expression conditions, proteins were collected from yeast media and purified by liquid chromatography. Only AtCKX7 protein that lacks a signal peptide and is presumably localized to cytosol was cloned into pPICZ vector and expressed intracellularly in *Pichia* after induction with methanol. Trials with pGAPZα vector were unsuccessful. However due to a troublesome purification from yeast cell lysate, AtCKX7 was expressed in *E. coli* with the use of pTYB12 vector that enables fast and easy purification.

Further large scale expression of AtCKX enzymes was established with the use of R‘ALF Plus Duet fermentor (10-litres working volume). Production of heterologous proteins in fermentor cultures results in a relatively high concentration of vacuolar proteases due to the combination of high-cell density and lysis of a small percentage of cells. *Pichia* strains deficient in vacuolar proteases should be used to reduce degradation of recombinant proteins. For that reason an auxotrophic and protease-deficient strain SMD1168 (*his4*, *pep4*) was chosen and new vector constructs were prepared. The expression cassette of pGAPZα vector containing constitutive GAP promoter and *N*-histagged *AtCKX* gene was inserted into *HIS4*-based vector pPIC9K. Resulting vectors gave higher expression levels of AtCKX proteins (at least two-fold increase) and therefore were selected for large scale expression in a

fermentor. Screening for CKX activity was done on the basis of best substrates and conditions for each enzyme, which were described before for plant extracts from transgenic tobacco plants overexpressing single *AtCKX* genes.

Non-secreted AtCKX proteins were characterized in the second part of this work. Recombinant enzymes after purification were subjected to SDS-PAGE and western blotting with polyclonal antibody raised against barley HvCKX2. Molecular masses of the studied proteins were also confirmed by MALDI-TOF intact protein mass measurement. In order to evaluate substrate specificity and electron acceptor preference activity measurements were performed for each enzyme. It was found that the activity of vacuolar AtCKX1 protein strongly depends on the presence of an appropriate electron acceptor in contrast to vacuolar AtCKX3 and cytosolic AtCKX7, which were less efficient dehydrogenases. Substrate specificity studies revealed that iP9G is the best substrate for AtCKX7 and one of the best substrates for AtCKX1 being rather poor for AtCKX3 that has no strong preference for any studied cytokinins. Interestingly, contrary to secreted CKX, all the non-secreted AtCKX proteins are able to effectively cleave all types of cytokinin nucleotides that have been proved as direct products of cytokinin biosynthesis in plants. Neither enzyme has preference for aromatic cytokinins. The recombinant enzymes were moreover identified by MALDI-TOF/MS.

The third part of this work is focused on the constitutive extracellular expression of AtCKX2 in *Pichia pastoris* under the GAP promoter with respect to a high-level production in fermentor cultures. The recombinant protein was fused with His-tag domain on its *C*-terminus, *N*-terminus and on both termini. However histidine fusions influenced enzymatic activity and the extent of such changes was determined.

Recombinant AtCKX2 enzyme was further used to develop cytokinin biosensor. Fabrication of such biosensor, the principle of its function and some preliminary data are presented in the last chapter of this work.

Aims of the work

1. Non-secreted cytokinin dehydrogenases of A*rabidopsis thaliana*: heterologous expression, purification and properties
    - To prepare constructs for directed secretion of vacuolar CKX enzymes
    - To purify and detect recombinant proteins
    - To characterize non-secreted AtCKX enzymes
    - To confirm the identity of recombinant proteins
2. High-level expression of recombinant cytokinin dehydrogenases from *Arabidopsis thaliana* in a constitutive *Pichia pastoris* expression system
    - To prepare aimed constructs for directed secretion and easy purification with the use of a polyhistidine domain
    - To determine the influence of fusion domains on enzymatic activity
    - To establish large scale proteins production in *Pichia pastoris* with the use of a fermentor
    - To develop cytokinin biosensor based on the AtCKX2 enzyme

Part 1

Introduction

Plant hormones cytokinins are N^6- substituted adenine derivatives bearing either an isoprenoid or an aromatic side chain. They were discovered in 1950s by Skoog and Miller who isolated kinetin (N^6-furfuryladenine) and described its ability to promote cell divisions in test tissues from tobacco (Miller *et al.,* 1955). Shortly after that it was proposed that cytokinins, together with auxins, play a major role in plant morphogenesis (Skoog and Miller, 1957). Considerable information has accumulated since then, concerning chemistry and physiology of cytokinins. It is known that those phytohormones serve as important signalling molecules in plant cells. They not only control cell division, but are involved in diverse events of plant growth and development, including apical dominance, shoot and root branching, leaf expansion, growth of lateral buds, photosynthesis, seed germination, floral transition and leaf senescence (Mok and Mok, 2001). Cytokinins also affect nutrient mobilization and biomass distribution (Takei *et al.,* 2001) and mediate a number of light-regulated processes, such as de-ethiolation and chloroplast differentiation during ripening (Mok, 1994). Moreover they participate in the maintenance of the meristem function (Kurakawa *et al.,* 2007) and response to environmental stimuli (Sakakibara *et al,* 2006; Werner *et al,* 2006). The content of endogenous cytokinins depends on the balance between de novo synthesis, import and export rate, interconversion among distinct forms, transient inactivation by conjugation (mainly glycosylation), and catabolic reactions resulting in a complete loss of biological activity (Sakakibara, 2006). The enzyme cytokinin dehydrogenase responsible for irreversible degradation of cytokinins (CKX, EC 1.5.99.12) was the objective of this study.

Cytokinin oxidase/dehydrogenase enzyme

The activity of CKX was first demonstrated in a crude tobacco culture by Pačes (Pačes *et al,* 1971) and later Whitty and Hall named the enzyme cytokinins oxidase (Whitty and Hall, 1974). The cytokinin degradation is achieved through oxidative cleavage of its N^6-side chain resulting in the formation of adenine and a side-chain derived aldehyde (Brownlee *et al*., 1975). The reaction proceeds via cytokinin dehydrogenation to an imine intermediate when an electron acceptor withdraws two electrons from the enzyme's flavin cofactor (Figure 1). For years it was assumed that molecular oxygen was essential for CKX activity but variety of electron acceptors other than oxygen, especially quinone-type, function more efficiently (Galuszka *et al,* 2001; Frébortová *et al.,* 2004). Therefore the enzyme was reclassified as dehydrogenase.

CKX is the only known enzyme shown to catalyze irreversible inactivation of cytokinins and its activity was reported in many plant species and in few lower organisms, namely the moss *Funaria hygrometrica* (Gerhäuser and Bopp, 1990), the slime mold *Dictyostelium discoideum* (Armstrong and Firtel, 1989) and the yeast *Sacharomyces cerevisiae* (Van Kast and Laten, 1987). However the presence of endogenous CKX in two latter organisms is doubtful since homologous gene sequences were not found (Schmülling *et al,* 2003).

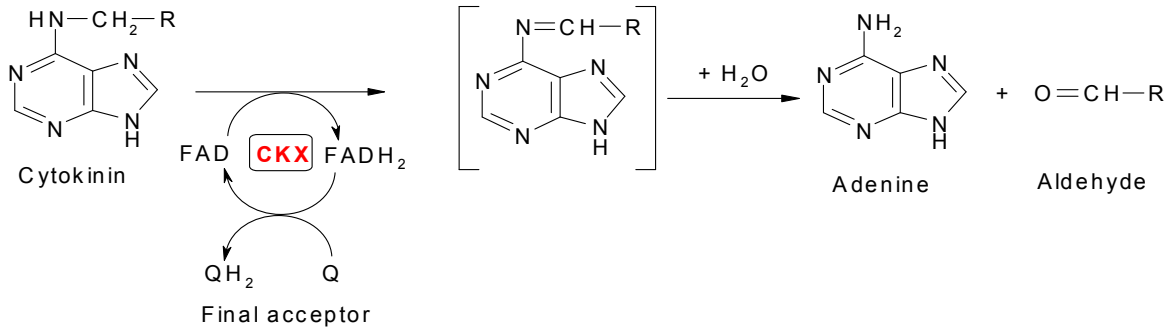


Figure 1. Scheme of cytokinin dehydrogenase reaction path.

Due to a low concentration of CKX in plant tissues the isolation of pure protein is extremely difficult. The breakthrough came with the cloning of a gene encoding CKX from maize (Houba-Hérin *et al.,* 1999; Morris *et al,* 1999) enabling identification of *CKX* genes in other species and further their expression in heterologous hosts or overexpression in plants. Genome-wide studies revealed the existence of small gene families coding for CKX in different higher plants (Werner *et al.,* 2006). The relationships between known CKX proteins from higher plants (*Zea mays*, *Oryza sativa*, *Arabidopsis thaliana*, *Hordeum vulgare*, *Dendrobium* hybrid "Sonia", *Dendrobium huoshanense*, *Triticum aestivum*, *Pisum sativum*, *Zinnia elegans*) and two putative proteins from cyanobacterium *Nostoc* sp. PCC 7120 (Kaneko *et al.,* 2001) and phytopathogen *Rhodococcus fascians* (Crespi *et al.,* 1994) could be presented by means of phylogenetic tree (Figure 2). Such analysis based on amino acids sequences suggests the same ancestral origin for all CKX members and it was proposed that plant genes might have been acquired through lateral transfer from bacteria to plants via the chloroplast, which is of cyanobacterial origin (Schmülling *et al,* 2003). In *Rhodococcus fascians* CKX is coded by *FasE* gene in the *fas* operon and recently its cytokinin-degrading activity was confirmed (Petry *et al*., 2010).

The common feature of all CKX enzymes is the presence of covalently bound FAD molecule. The cofactor is linked through the 8-methyl group of the isoalloxazine ring to a histidine residue of well conserved GHS motif in the enzymes' *N*-terminal halves (Malito *et al.,* 2004). Covalent binding of FAD to protein facilitates redox catalysis and the oxidative power of flavin (Galuszka *et al.,* 2008). The only known exception is *Nostoc* CKX that contains GYT motif. Nevertheless tyrosine is able to bind FAD as well (Mewies *et al*., 1998) and the presence of the flavin cofactor in NsCKX was confirmed by UV/VIS and fluorescent spectra (Frébortová *et al*., unpublished).

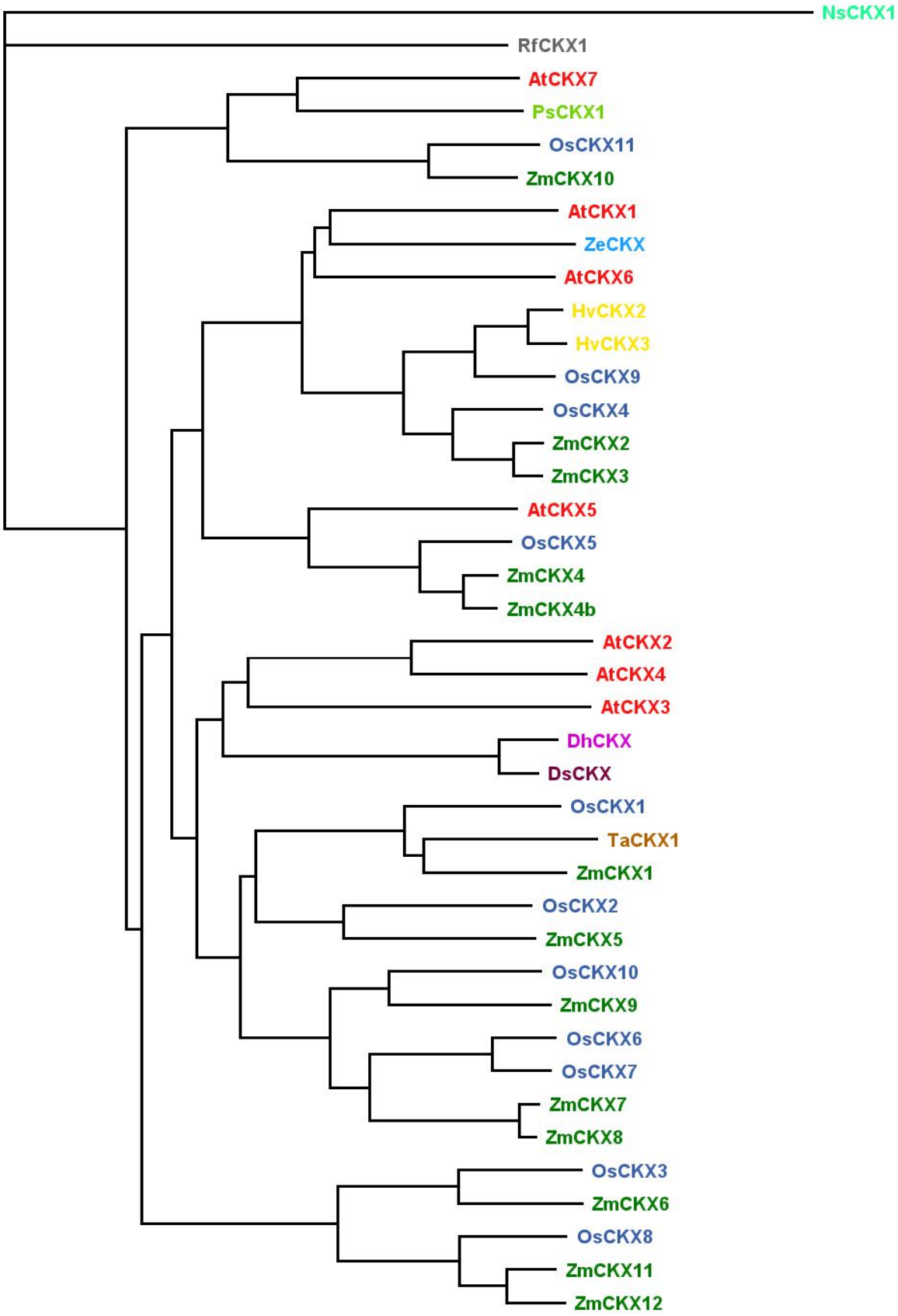


Figure 2. Neighbour-joining phylogenetic tree of the known and putative CKX amino acid sequences (Geneious 5.0.2). Branch distances correspond to sequence divergence.

Mature CKX enzymes consist of two domains for FAD and substrate binding, respectively. About one-third of all amino acids positions are highly conserved among different CKX proteins. The FAD-binding domain contains large part of conserved regions but short highly conserved sequences are also located at the *N*- and *C*-termini of this motif. Other examples include WevPHPWLNl sequence found around position 390 or PGQxIF signature at the *C*-terminal ends of the proteins (Schmülling *et al,* 2003). Conservation of amino acid residues suggests their functional importance possibly in substrates recognition and electron transport.

Cytokinin dehydrogenases from Arabidopsis thaliana

A family of seven homologous genes coding for CKX is present in *Arabidopsis thaliana* genome. Two genes are located on chromosome 2 (designated *AtCKX1* and *AtCKX2*), two on chromosome 5 (*AtCKX3* and *AtCKX7*) and single genes are found on chromosomes 4, 1 and 3 (*AtCKX4*, *AtCKX5* and *AtCKX6*, respectively). The genomic organisation of all sequences is highly conserved with five exons and four introns (Bilyeu *et al.,* 2001).

The predicted proteins are of similar size (~60 kDa) but their amino acid identity is between 34.3% (AtCKX4 and AtCKX7) and 65.9% (AtCKX2 and AtCKX4). Sequence alignment (Figure 3) indicates conservation of FAD-binding site and the presence of several small domains as mentioned above. The divergences in amino acid sequences contribute to functional diversification of AtCKX isoenzymes. Except for AtCKX7, all proteins have hydrophobic *N*-terminal signal peptide (SignalP 3.0 Server, Bendtsen *et al,* 2004). Four proteins (AtCKX2, AtCKX4, AtCKX5 and AtCKX6) are targeted to apoplast and the remainder (AtCKX1 and AtCKX3) are found in vacuoles, despite their mitochondrial prediction. Secretion of AtCKX2 was confirmed by GFP-fusion as well as vacuolar localisation of AtCKX1 and AtCKX3 (Werner *et al.,* 2003). The lack of signal peptide in the case of AtCKX7 indicates its cytosolic localisation what is in agreement with the intracellular protein expression in *E.coli* or *P.pastoris* (Kowalska, unpublished).

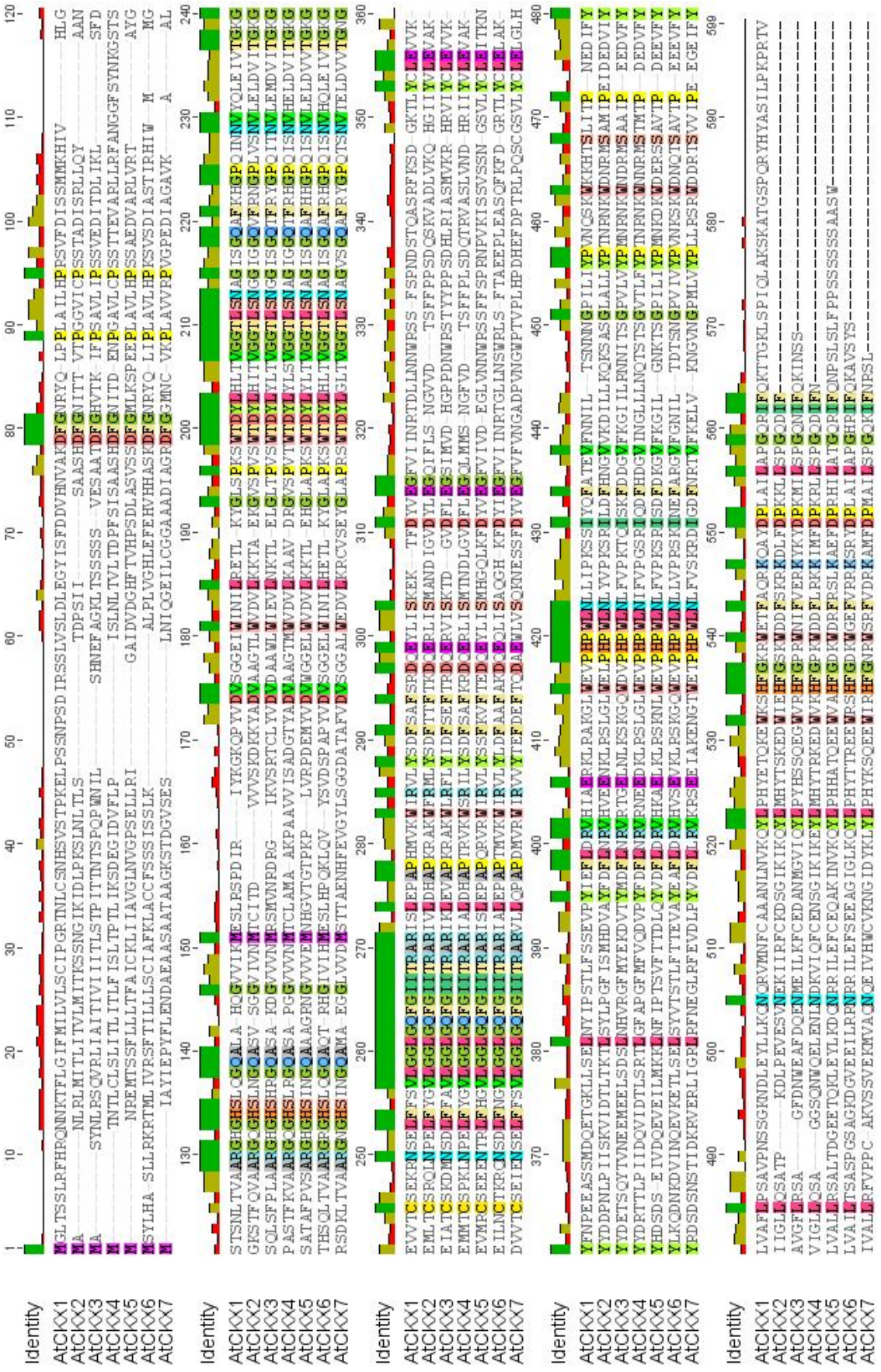


Figure 3. Amino acid sequence alignment of AtCKX proteins (Geneious 5.0.2).

Table 1. Features of AtCKX genes and proteins.

Gene name	Accession number	Chromosome	Length (aa)	Mass* (kDa)	Isoelectric point*	Glycosylation sites♦
AtCKX1	AT2G41510	2	575	64.9	9.76	8/5
AtCKX2	AT2G19500	2	501	55.5	7.67	3/3
AtCKX3	AT5G56970	5	523	59.4	6.80	4/2
AtCKX4	AT4G29740	4	524	58.1	6.19	4/4
AtCKX5	AT1G75450	1	540	60.4	6.42	3/2
AtCKX6	AT3G63440	3	533	60.0	9.11	4/4
AtCKX7	AT5G21482	5	524	57.9	4.78	6/6

*Molecular masses and isoelectric points were calculated in Geneious 5.0.2.

♦All predicted glycosylation sites/predicted glycosylation sites with more than 50% probability; calculated with NetNGly (http://www.cbs.dtu.dk/services/NetNGlyc). However intracellular proteins are unlikely to be glycosylated.

AtCKX proteins are posttranslationally modified by glycosylation since they contain several consensus N-glycosylation sites (Asn-X-Ser/Thr; Table 1). Such modification contributes to regulation of enzymatic activity (different pH optimum), translocation and protein stability (Schmülling *et al,* 2003) but is not necessary for correct protein folding as was found out by expression of active ZmCKX proteins in *E. coli* (Zalabák, unpublished). Also, the occurrence of both glycosylated and non-glycosylated isoforms of CKX was confirmed in various plant sources and cultured tobacco callus, with higher activity of the glycosylated enzyme (Kamínek and Armstrong, 1990; Motyka *et al.,* 2003).

Arabidopsis cytokinin dehydrogenase structure and consequent substrate interactions

The crystal structure of AtCKX7 was recently determined (Figure 4; Wesenberg *et al.,* unpublished; Bae *et al.,* 2008). Although the protein shares only 39.4% amino acids identity with ZmCKX1, the structures of both enzymes are very similar, showing highly conserved active site. AtCKX7 structure comprises 18 β-strands and 2 β-bridges, 14 α-helices and seven 3-10 helices. The protein displays two-domain folding topology typical for the members of the vanillyl-alcohol oxidase family (Malito *et al.,* 2004). The ADP moiety of the cofactor is embedded in the FAD-binding domain while its isoalloxazine ring is located at the interface of the two domains. The 8-methyl group of the flavin is covalently linked to the conserved His96 residue with a distance of 1.4 Å (Bae *et al,* 2008). The CKX active site consists of an internal cavity lined by the flavin ring and a funnel-shaped region on the protein surface. Due to the bipartite nature of enzyme's active site, cytokinin interacts with

CKX in a plug-into-socket mode. Aliphatic side-chain of the substrate is sealed in solvent inaccessible cavity where it interacts with Asp162, Trp382 and Leu443 residues in the proximity of FAD. Funnel-shaped region is the binding site for the adenine ring that is sandwiched between non-polar residues Val363 and Pro411 but its edge is exposed near the protein surface to the solvent. It was proposed that Asp162 is the only H-bond acceptor for the substrate amine group and its interaction with Glu275 (carboxylate-carboxylate pair) stabilizes the neutral form of the bound substrate. All mentioned residues are highly conserved except for Pro411 that in AtCKX2 and AtCKX4 is replaced by Leu415 and Val426, respectively (Galuszka *et al.,* 2007). However significant alteration in one amino acid residue was found when comparing to ZmCKX1 structure. In the active site of the maize enzyme there is Glu residue (Glu381) confirmed to form a hydrogen bond with the N9 atom of the adenine ring of N^6-(2-isopentenyl)adenine (iP). This residue is conserved only in the sequences of AtCKX2 (Glu348) and AtCKX4 (Glu371), whereas in other enzymes it is substituted by serine (AtCKX6 – Ser372, AtCKX7 – Ser366), alanine (AtCKX1 – Ala385, AtCKX5 – Ala366) or glycine (AtCKX3 – Gly366) (Galuszka *et al.,* 2007). Surprisingly, in the active site of CKX there is no room for an electron acceptor to bind in the vicinity of FAD. Therefore it is assumed that electrons are transferred from the flavin through the protein matrix to an electron acceptor bound on the protein surface. However it is unknown how electron acceptors of CKX function *in vivo*.

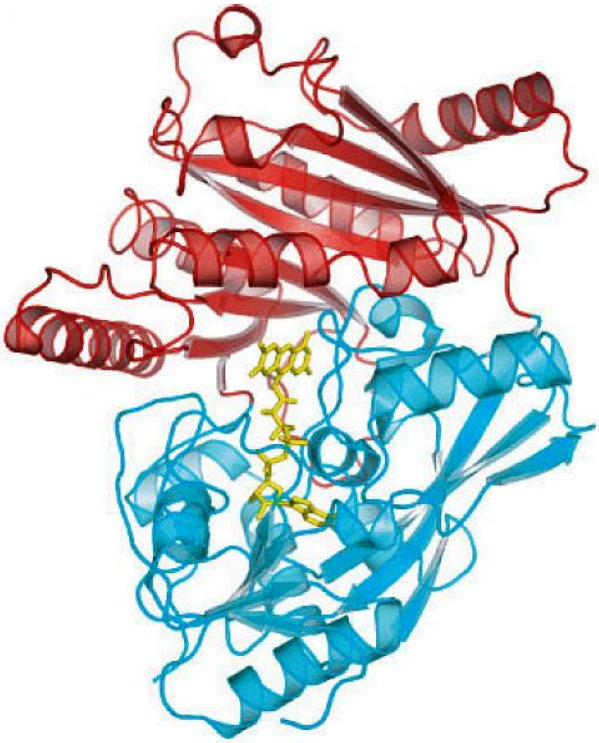


Figure 4. Ribbon diagram of AtCKX7 crystal structure (PDB entry 2EXR). The FAD-binding and the substrate-binding domains are shown in cyan and red, respectively. The FAD molecule is represented as a stick model.

Properties of AtCKX enzymes

Functional diversification of *Arabidopsis thaliana* CKX isoenzymes is also manifested through different gene expression patterns and biochemical properties. The expression of individual *AtCKX* genes at different developmental stages and in different plant tissues was confirmed using fusion constructs of *CKX* promoter regions and β-glucuronidase (GUS; Werner *et al.,* 2003). AtCKX1 showed prominent expression in the vascular cylinder of lateral roots, whereas AtCKX2 showed strongest expression in the shoot apex, and AtCKX3 was expressed in the young shoot tissues. AtCKX4 had interesting pattern of expression in developing trichomes, stomata and stipules, as well as the root cap – mostly regions of high mitotic activity. Besides differential patterns of expression in developing shoots and roots, AtCKX5 showed strong expression in stamen primordial and developing pollen, while AtCKX6 was expressed in the gynoecium at various stages of development. Subtle variation in the amino acid residues of enzymes' active site (as mentioned above) possibly contributes significantly to the substrate binding and turnover rates of individual substrates. The substitution of glutamic acid in the active site (maize Glu381) with hydrophobic residues or serine can cause less tight binding of the free cytokinin base i. e. lower activity with iP. Indeed, AtCKX2 and AtCKX4, that possess the Glu residue, are the most active and in neutral or slightly basic pH prefer free cytokinin bases as substrates (Galuszka *et al.,* 2007). Substrate specificity is also thought to be closely related to the enzymes' subcellular localization.

Constitutive expression of *AtCKX* genes in tobacco and *Arabidopsis thaliana* resulted in cytokinin-deficient plants (30-60% of wild type cytokinin content) that typically exhibited stunted shoots with smaller apical meristems, prolonged plastochrone, slower leaf cell production and excessive root system development (Werner *et al.,* 2001 and 2003). Phenotypes of plants overexpressing individual *AtCKX* genes differ to some extent, especially the overexpression of vacuolar proteins caused stronger cytokinin deficiency syndrome than the overexpression of apoplastic enzymes. Taken together, these facts indicate that specific developmental and physiological functions are fulfilled by each *AtCKX* gene and that the tissue-specific regulation of the endogenous cytokinin content is important for ensuring the proper regulation of cytokinin functions in plants (Werner *et al.,* 2003).

CKX functions and possible applications

Under normal growth conditions CKX enzymes maintain the homeostasis of endogenous cytokinin levels required for plant growth and development. Also, hormone degradation could be the way how to reset the cytokinin-sensing system to a basal level, so the function of CKX would be the control of cytokinin concentrations available to the plasma membrane receptors (Bürkle *et al.,* 2003). In cell divisions zones, CKX may be relevant for the recovery of the purine moiety of the hormone

and degradation of cell cycle-derived cytokinins (Werner *et al.,* 2003). For example, AtCKX1 preferentially cleaves N^6-glucosides, which normally accumulate in vacuoles, therefore the enzyme recycles components of cytokinin molecules (Galuszka *et al.,* 2007). It was proposed that each cell during division produces its own cytokinin and AtCKX may serve as protectant from cytokinins derived from neighboring cells, preserving the cytokinin autonomy of each single cell (Werner *et al.,* 2003). These enzymes also seem to isolate reproductive tissues from the activity of translocated cytokinins (Bilyeu *et al.,* 2003). Moreover, a role of CKX in some tissues could be the control of sink strength (Werner *et al.,* 2003). Cytokinin dehydrogenases are therefore responsible for fine-tuning of cytokinin level at precise locations. Additionally, in rice CKXs regulate grain production and in maize they possibly protect the kernel from invasion of fungal pathogens and prevent precocious germination (Ashikari *et al.,* 2005; Schmülling *et al,* 2003).

Targeted manipulation of *CKX* gene expression will help clarify the specific functions of cytokinins. But most importantly, since CKX creates numerous possibilities for the precise control of endogenous cytokinins it can be used to modulate growth characteristics and improve agricultural traits of crop plants.

References

Armstrong, D.J., Firtel, R.A., 1989. Cytokinin oxidase activity in the cellular slime mold *Dictyostelium discoideum.* Dev. Biol. 136, 491–499.

Ashikari,M., Sakakibara, H., Lin, S., Yamamoto, T., Takashi, T., Nishimura, A., Angeles, E.R., Qian, Q., Kitano, H., Matsuoka, M., 2005. Cytokinin oxidase regulates rice grain production. Science 309, 741-745.

Bae, E., Bingman, C.A., Bitto, E., Aceti, D.J., Philips Jr., G.N., 2008. Crystal structure of *Arabidopsis thaliana* cytokinin dehydrogenase. Proteins 70, 303-306.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783-795.

Bilyeu, K.D., Cole, J.L., Laskey, J.G., Riekhof, W.R., Esparza, T.J., Kramer, M.D., Morris, R.O., 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol. 125, 378-386.

Bilyeu, K.D., Laskey, J.G., Morris, R.O., 2003. Dynamics of expression and distribution of cytokinin oxidase/dehydrogenase in developing maize kernels. Plant Growth Regul. 39, 195-203.

Brownlee, B.G., Hall, R.H., Whitty, C.D., 1975. 3-Methyl-2-butenal: an enzymatic degradation product of the cytokinin N^6-(Δ^2-isopentenyl)adenine. Can. J. Biochem. 53, 37-41.

Bürkle, L., Cedzich, A., Döpke, C., Stransky, H., Okumoto, S., Gillissen, B., Kühn, C., Frommer, W.B., 2003. Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of *Arabidopsis.* Plant J., 34, 13-26.

Crespi, M,. Vereecke, D., Temmerman, W., Van Montagu, M., Desomer, J., 1994. The *fas* operon of *Rhodococcus fascians* encodes new genes required for efficient fasciation of host plants. J. Bacteriol. 176, 2492–2501.

Frébortová, J., Fraaije, M.W., Galuszka, P., Šebela, M., Peč, P., Hrbáč, J., Bilyeu, K.D., English, J.T., Frébort, I., 2004. Catalytic reaction of cytokinin dehydrogenase: Preference for quinones as electron acceptors. Biochem. J. 380, 121-130.

Galuszka, P., Frébort, I., Šebela, M., Sauer, P., Jacobsen, S., Peč, P., 2001. Cytokinin oxidase or dehydrogenase? Mechanism of the cytokinin degradation in plants. Eur. J. Biochem. 268, 450-461.

Galuszka, P., Popelková, H., Werner, T., Frébortová, J., Pospíšilová, H., Mik, V., Köllmer, I., Schmülling, T., Frébort, I., 2007. Biochemical characterization and histochemical localization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabaccum* L. J. Plant Growth Regul. 26, 255-267.

Galuszka, P., Spíchal, L., Kopečný, D., Tarkowski, P., Frébortová, J., Šebela, M., Frébort, I., 2008. Metabolism of plant hormones cytokinins and their function in signaling, cell differentiation and

plant development. In Studies in Natural Products Chemistry, vol. 34 (Atta-ur-Rahman, ed.) pp. 203-264, Elsevier, Amsterdam, The Netherlands.

Gerhäuser, D., Bopp, M., 1990. Cytokinin oxidases in mosses. 2. Metabolism of kinetin and benzyladenine *in vivo*. J. Plant Physiol. 135, 714–718.

Houba-Hérin, N., Pethe, C., d'Alayer, J., Laloue, M., 1999. Cytokinin oxidase from *Zea mays*: purification, cDNA cloning and expression in moss protoplasts. Plant J. 17, 615-626.

Kamínek, M., Armstrong, D.J., 1990. Genotypic variation in cytokinin oxidase from *Phaseolus* callus cultures. Plant Physiol. 93, 1530–1538.

Kaneko, T., Nakamura, Y., Wolk, C.P., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kohara, M., Matsumoto, M., Matsuno, A., Muraki, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takazawa, M., Yamada, M., Yasuda, M., Tabata, S., 2001. Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 (supplement). DNA Res. 8, 205–213.

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

Malito, E., Coda, A., Bilyeu, K.D., Fraaije, M.W., Mattevi, A., 2004. Structures of Michaelis and product complexes of plant cytokinin dehydrogenase: Implications for flavoenzyme catalysis. J. Mol. Biol. 341, 1237-1249.

Mewies, M., McIntire, W. S., Scrutton, N. S., 1998. Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: the current state of affairs. Protein Sci. 7(1), 7-20.

Miller, C.O., Skoog, F., Von Saltza, M.H., Strong, F., 1955. Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. 77, 1392.

Mok, D.W., Mok, M.C., 2001. Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 89-118.

Mok, M.C., 1994. Cytokinins and plant development: An overview. In Cytokinins: Chemistry, Activity, and Function (D.W.S. Mok and M.C. Mok, eds) pp. 155–166, CRC Press, Boca Raton, FL, USA.

Morris, R.O., Bilyeu, K.D., Laskey, J.G., Cheikh, N.N., 1999. Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. Biochem. Biophys. Res. Commun. 255, 328-333.

Motyka, V., Vaňková, R., Čapková, V., Petrášek, J., Kamínek, M., Schmülling, T., 2003. Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. Physiol. Plant 117, 11-21.

Pačes, V., Werstiuk, E., Hall, R.H., 1971. Conversion of N^6-(Δ^2-isopentenyl)adenosine to adenosine by enzyme activity in tobacco tissue. Plant Physiol. 48, 775-778.

Pertry, I., Václavíková, K., Gemrotová, M., Spíchal, L., Galuszka, P., Depuydt, S., Temmerman, W., Stes, E., De Keyser, A., Riefler, M., Biondi, S., Novák, O., Schmülling, T., Strnad, M., Tarkowski, P., Holsters, M., Vereecke, D., 2010. *Rhodococcus fascians* impacts plant development through the dynamic Fas-mediated production of a cytokinin mix. Mol. Plant Microbe Interact., in press

Sakakibara H., 2006. Cytokinins: Activity, biosynthesis, and translocation. Annu. Rev. Plant Biol. 57, 431-439.

Schmülling, T., Werner, T., Riefler, M., Krupková, E., Bartrina y Manns, I., 2003. Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. J. Plant Res. 116, 241-252.

Skoog, F., Miller, C.O., 1957. Chemical regulation of growth and organ formation in plant tissue cultured in vitro. Symp. Soc. Exp. Biol. XI, 118–131.

Takei, K, Sakakibara, H, Taniguchi, M, Sugiyam, T., 2001. Nitrogen‐dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. Plant Cell Physiol. 42, 85–93.

Van Kast, C.A., Laten, H., 1987. Cytokinin utilization by adenine requiring mutants of the yeast *Saccharomyces cerevisiae*. Plant Physiol. 83, 726–727.

Werner, T., Motyka, V., Laucou, V., Smets, R., van Onckelen, H., 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15, 1-20.

Werner, T., Köllmer, I., Bartrina y Manns, I., Holst, K., Schmülling, T., 2006. New insights into the biology of cytokinin degradation. Plant Biol. 8, 371-381.

Whitty, C.D., Hall, R.H., 1974. A cytokinin oxidase in *Zea mays*. Can. J. Biochem. 52, 787–799.

Part 2

Vacuolar and cytosolic cytokinin dehydrogenases of *Arabidopsis thaliana*: Heterologous expression, purification and properties

Marta Kowalska[a,*], Petr Galuszka[a], Jitka Frébortová[b], Marek Šebela[a], Tibor Béres[b], Tomáš Hluska[a], Mária Šmehilová[a], Kristin D. Bilyeu[c], Ivo Frébort[a]

[a]*Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic*
[b]*Laboratory of Growth Regulators, Faculty of Science, Palacký University and Institute of Experimental Botany of the Academy of Science, Šlechtitelů 11, 783 71 Olomouc, Czech Republic*
[c]*United States Department of Agriculture, Agricultural Research Service, Plant Genetics Research Unit, 110 Waters Hall, Columbia, MO 65211, USA*

*Corresponding author. Tel.: +420 585 634 923; fax: +420 585 634 936.
E-mail address: marta.kowalska@email.cz

Phytochemistry (2010); doi:10.1016/j.phytochem.2010.08.013

Abstract

The catabolism of cytokinins is a vital component of hormonal regulation, contributing to the control of active forms of cytokinins and their cellular distribution. The enzyme catalyzing the irreversible cleavage of N^6-side chains from cytokinins is a flavoprotein classified as cytokinin dehydrogenase (CKX, EC 1.5.99.12). CKXs also show low cytokinin oxidase activity, but molecular oxygen is a comparatively poor electron acceptor. The *CKX* gene family of *Arabidopsis thaliana* comprises seven members. Four code for proteins secreted to the apoplast, the remainder are not secreted. Two are targeted to the vacuoles and one is restricted to the cytosol. This study presents the purification and characterization of each of these non-secreted CKX enzymes and substrate specificities are discussed with respect to their compartmentation. Vacuolar enzymes AtCKX1 and AtCKX3 were produced in *Pichia pastoris* and cytosolic enzyme AtCKX7 was expressed in *Escherichia coli*. The recombinant proteins were purified by column chromatography. All enzymes preferred synthetic electron acceptors over oxygen, namely potassium ferricyanide and 2,3-dimetoxy-5-methyl-1,4-benzoquinone (Q_0). In slightly acidic conditions (pH 5.0), N^6-(2-isopentenyl)adenine 9-glucoside (iP9G) was the best substrate for AtCKX1 and AtCKX7, whereas AtCKX3 preferentially degraded N^6-(2-isopentenyl)adenine 9-riboside-5'-monophosphate (iPMP). Moreover, vacuolar AtCKX enzymes in certain conditions degraded N^6-(2-isopentenyl)adenine di- and triphosphates two to five times more effectively than its monophosphate.

***Keywords*:** *Arabidopsis thaliana*; *Pichia pastoris* expression system; Electron acceptor; Substrate specificity; Vacuolar sorting signal; Cytokinin dehydrogenase.

***Abbreviations*:** ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); AtCKX, cytokinin dehydrogenase from *Arabidopsis thaliana*; cZ, *cis*-zeatin; HvCKX, cytokinin dehydrogenase from *Hordeum vulgare*; iP, N^6-(2-isopentenyl)adenine; iP9G, N^6-(2-isopentenyl)adenine 9-N-glucoside; iPDP, N^6-(2-isopentenyl)adenine 9-riboside-5'-diphosphate; iPMP, N^6-(2-isopentenyl)adenine 9-riboside-5'-monophosphate; iPR, N^6-(2-isopentenyl)adenine 9-riboside; IPTG, Isopropyl β-D-1-thiogalactopyranoside; iPTP, N^6-(2-isopentenyl)adenine 9-riboside-5'-triphosphate; tZ, *trans*-zeatin; YPD, yeast extract peptone dextrose medium; ZDP, zeatin 9-riboside-5′-diphosphate; ZMP, zeatin 9-riboside-5′-monophosphate; ZTP, zeatin 9-riboside-5′- triphosphate; iPR, N^6-(2-isopentenyl)adenine 9-riboside

Introduction

Cytokinins are important signaling molecules regulating cell division and differentiation in plant cells (Mok and Mok, 2001). Not all forms of cytokinins are biologically active but it is likely that the concentrations of both active and inactive cytokinins are actively managed. Concentrations of active cytokinins and their distribution in plant tissues are controlled by irreversible degradation (Hare and van Staden, 1994; Galuszka *et al*., 2000) as well as through synthesis and compartmentation. The enzyme playing a key role in cytokinin catabolism is cytokinin dehydrogenase (CKX; EC 1.5.99.12), a flavoprotein that cleaves off the N^6-side chain of cytokinins to produce a corresponding aldehyde (Brownlee *et al*., 1975). CKX activity was first described in 1971 in a crude tobacco cell culture extracts (Pačes *et al*., 1971). For many years the enzyme had been classified as an oxidase, but more recent findings indicated that electron acceptors other that oxygen are preferred (Galuszka *et al*., 2001; Bileyu *et al*., 2001; Laskey *et al*., 2003), therefore the enzyme was reclassified as dehydrogenase.

In higher plant species CKX proteins are encoded by small gene families with a varying number of members (Werner *et al*., 2006). Analysis of the entire genome of *Arabidopsis thaliana* has revealed seven distinct CKX-encoding genes (*AtCKX1-AtCKX7*). Amino acids identity among individual AtCKX proteins is between 34.3 and 65.9% (Schmülling *et al*., 2003). The proteins share conserved FAD-binding and substrate-binding domains, but the sequences outside these domains display strong divergence (Popelková *et al*., 2004). AtCKX enzymes differ in their biochemical properties, in regulation of their expression and in subcellular localization (Schmülling *et al*., 2003). *N*-terminal sequences of AtCKX proteins indicate that four of them (AtCKX2, AtCKX4, AtCKX5 and AtCKX6) are targeted to the plant secretory pathway, whereas AtCKX7 is probably localized to the cytosol due to the lack of any recognized signal peptide. Finally AtCKX1 and AtCKX3 were initially predicted to be imported into mitochondria (TargetP and PSORT programs, Emanuelsson *et al*. (2000) and Nakai *et al*. (1999), respectively), but the study of their GFP fusion proteins confirmed their presence in vacuoles (Werner *et al*., 2003).

Purification of CKX enzymes from natural sources like maize (Morris *et al*., 1999), wheat or barley (Galuszka *et al*., 2001) posed an extreme challenge and required several advanced purification steps. In general CKX proteins show low retention on chromatographic columns and are difficult to concentrate by salting out. Fortunately they possess very stable activity. Recombinant CKX enzymes from *A. thaliana* were individually expressed in transgenic tobacco plants (Werner *et al*., 2001) and the plant extracts examined for CKX activity and substrate specificity (Galuszka *et al*., 2007). Heterologous expression of AtCKX2, the most abundant secreted *Arabidopsis* CKX enzyme, has been achieved in *Sacharomyces cerevisiae* (Werner *et al*., 2001; Frébortová *et al*., 2007) and *Pichia pastoris* (Bilyeu *et al*., 2001) allowing purification and biochemical characterization (Frébortová *et al*., 2007).

Non-secreted AtCKX enzymes have not previously been studied thoroughly. The crystal structure of cytosolic AtCKX7 protein has been reported (Bae *et al*., 2008) as an output from an eukaryotic structural genomics pipeline, but its activity or catalytic properties were not investigated. In this work, recombinant AtCKX7 was obtained from *E. coli* BL21 (DE3) STAR lysate and vacuolar proteins AtCKX1 and AtCKX3 were purified from *P. pastoris*, using directed secretion to the culture medium. We determined the properties of all three enzymes purified to homogeneity.

Results and discussion

Targeting of AtCKX proteins to vacuoles

AtCKX proteins have different subcellular localizations and earlier work has shown that AtCKX1 (GenBank accession No. NP_181682) and AtCKX3 (NP_200507) are vacuolar (Werner *et al*., 2003) by means of GFP fusions in transgenic *A. thaliana* plants. It was supposed that vacuolar targeting might be specific to certain cell types since AtCKX3 was found mainly in central vacuoles, whereas AtCKX1 only in smaller vacuoles (Werner *et al*., 2003). As part of the *P.pastoris* expression strategy, the sequence of AtCKX3 was examined for targeting motifs (SignalP 3.0 Server, Bendtsen *et al*., 2004). Initial expression of AtCKX3, however, was not efficient and no activity was detected in the transformed yeast culture. Further sequence analysis of AtCKX3 revealed the presence of a sequence similar to an *N*-terminal sequence-specific vacuolar sorting signal (ssVSS). This motif targets proteins to lytic vacuoles; it is typically present in the protein after the *N*-terminal signal peptide (a secretory pathway signal) and contains the degenerate amino acid sequence [N/L]-[P/I/L]-[I/P]-[R/N/S], also called the NPIR consensus (Nakamura and Matsuoka, 1993; Vitale and Raikhel, 1999). When a re-designed gene construct was prepared, AtCKX3 recombinant protein with a yeast signal peptide and the vacuolar sorting signal deleted was properly processed by the yeast and secreted to the medium in its active form. The NPIR consensus was also found in the sequence of AtCKX1 (Figure 1) and deleted before the expression in *Pichia*. The activity of the NPIR motif in yeast complements the earlier GFP data to confirm that these CKX isoforms are directed to vacuoles. Deletion of the motif was essential for satisfactory expression in yeast.

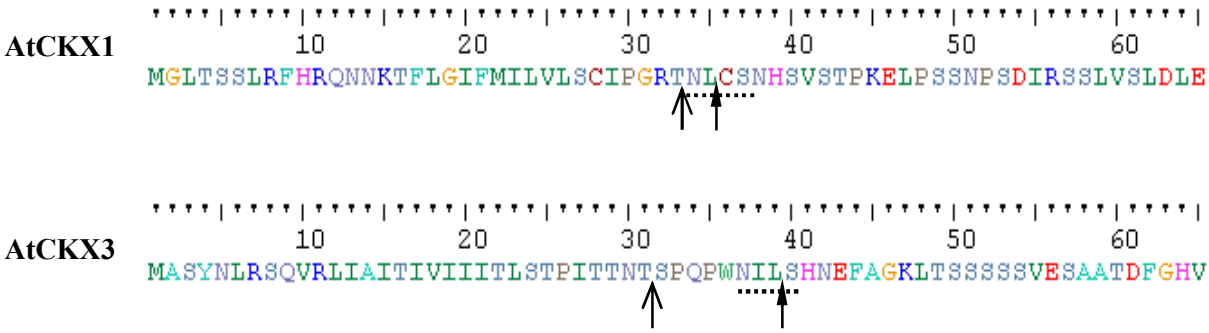


Figure 1. Identification of the vacuolar targeting sequence in AtCKX1 and AtCKX3. *N*-terminal sequence-specific vacuolar sorting signal (ssVSS) that typically it contains degenerate signal [N/L]-[P/I/L]-[I/P]-[R/N/S] called also NPIR consensus was found. The ssVSS is underlined by a dotted line and the arrows indicate the predicted end of a signal peptide sequence (SignalP 3.0 Server; thin arrows) and a peptide sequence that had to be cleaved to achieve secretion of active proteins from the yeast cells (thick arrows), respectively.

Selection of vectors and expression system

Vacuolar AtCKX enzymes were first expressed in pGAPZα vector to confirm the protein secretion to *Pichia* growth medium by CKX activity assay. In order to facilitate purification, recombinant proteins were fused with a *C*-terminal His-tag domain. Initial experiments showed that *C*-terminal His-tag fusion (in pGAPZα) decreases CKX specific activity (67% activity loss), therefore a novel vector was prepared from pGAPZαA by inserting a coding sequence for 10x His between the α-factor coding sequence and the multiple cloning site. *AtCKX* genes were subsequently cloned into pGAPZαA$(His)_{10}$ and expressed in *Pichia pastoris* X-33. Expression of heterologous proteins in fermentor cultures can result in accumulation of vacuolar proteases such as proteinase A (*pep4*) with resulting damage to the protein of interest (Lin-Cereghino and Lin-Cereghino, 2007). For that reason an auxotrophic and protease-deficient strain SMD1168 (*his4*, *pep4*) was chosen and new vector constructs were prepared. The expression cassette of pGAPZαA$(His)_{10}$::AtCKX containing constitutive GAP (glyceraldehyde 3-phosphate dehydrogenase) promoter and *AtCKX* gene was inserted into *HIS4*-based vector pPIC9K (Figure 2). The resulting vectors gave acceptable expression levels of vacuolar AtCKX proteins and were selected for large scale expression in a fermentor.

Cytosolic enzyme AtCKX7 was expressed intracellularly in *P. pastoris* in a shake flask but the protein purification from lysate was very difficult and insufficient (data not shown). Therefore expression in *E. coli* was chosen with the vector pTYB12 that introduces a chitin-binding intein tag to enable fast and easy purification.

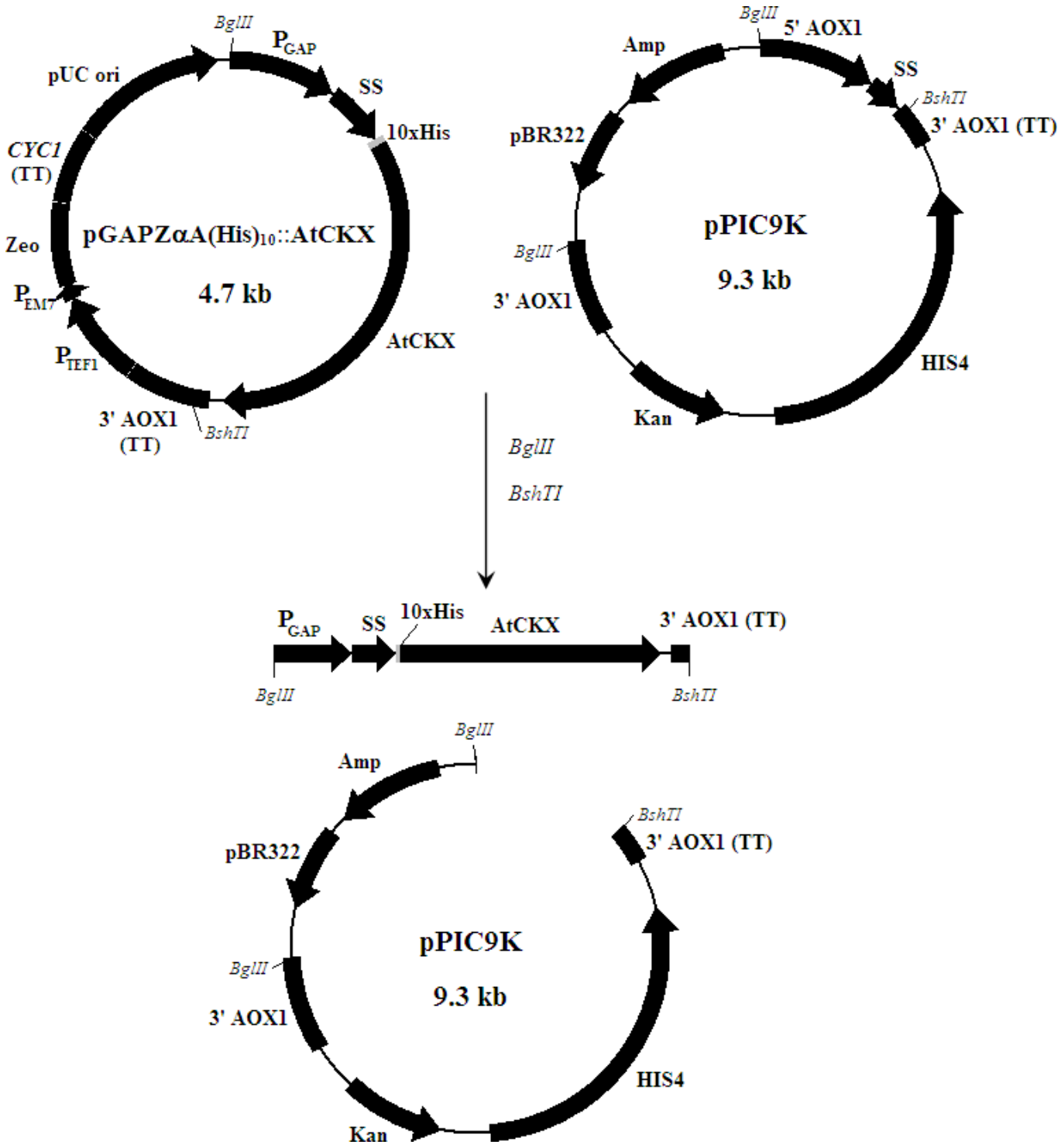


Figure 2. Preparation of pPIC9K vector under the control of constitutive GAP promoter for expression of AtCKX proteins. pPIC9K vector and proper plasmid construct pGAPZαA(His)$_{10}$::AtCKX were treated by restriction enzymes *BglII* and *BshTI*. After partial digestion the products of expected size were selected (approx. 8 kb for pPIC9K and 2.4-2.5 kb for pGAPZαA(His)$_{10}$::AtCKX) to be further ligated together and used for *E. coli* transformation.

Production of recombinant AtCKX proteins

AtCKX1 and *AtCKX3* genes were expressed in *P. pastoris* and active proteins were released into the growth medium. Screening for activity used assay conditions described earlier (Galuszka *et al*., 2007). Yeasts were grown first in 250 ml Erlenmeyer flasks to confirm CKX activity and then the process was scaled up using a fermentor. After fermentation, approximately 8 l of culture media were concentrated by ultrafiltration and purified by column chromatography (Table 1). Ammonium sulfate precipitation was ineffective, even at 100% saturation, probably due to the presence of a silicone antifoam agent that was added in the fermentor. First chromatographic step (Octyl Sepharose or hydroxyapatite for AtCKX1 and AtCKX3, respectively) resulted in low protein recovery since much CKX was washed out from the column together with the antifoam agent during an isocratic wash at the start of the chromatographic run. Ni affinity chromatography was ineffective at early stage and later on gave only moderate purification. 95% pure AtCKX1 was eluted from a Hema-Bio Phenyl column and AtCKX3 was purified on Resource Q column.

In general, purification of vacuolar AtCKX proteins was problematic. The use of the silicone antifoam agent was essential in the fermentor, but compromised column chromatography. The proteins also exhibited low polarity. Overall yields were low (1.3 and 0.5% for AtCKX1 and AtCKX3, respectively), but acceptable. Similar difficulties caused by the low polarity of CKX were previously described for recombinant AtCKX2 (Frébortová *et al*., 2007) and for enzymes from wheat and barley (Galuszka *et al*., 2001). It was not the case for recombinant ZmCKX1 (Bilyeu *et al*., 2001; Kopečný *et al*., 2005) therefore it is suspected that low polarity of CKX may be related to the amino acid sequence, the structure of the protein and posttranslational modifications (Frébortová *et al*., 2007).

Cytosolic AtCKX7 protein was expressed in *E. coli* with IPTG induction. The bacterial cells were disrupted using a French press and the enzyme was purified from lysate (50-fold, 33% recovery) in a single step on a chitin resin column, giving a protein with an activity of 181 nkat/mg with 250 μM iP in pH 7.0. In 2008, the crystal structure of AtCKX7 was reported (without measuring any data on enzymatic activity); the protein was prepared by the expression in CESG vector pVP13-GW, where the protein was bearing *N*-terminal $(His)_6$-MEB fusion tag enabling a generic Ni-IMAC purification strategy. The 33 *N*-terminal residues were not assigned in the model (Bae *et al*., 2008).

The purity of each AtCKX protein was examined by SDS-PAGE (Figure 3). AtCKX1 and AtCKX3 proteins migrated as single sharp bands corresponding to molecular mass of approximately 97 kDa and 92 kDa, respectively, whereas AtCKX7 gave one sharp band of molecular mass of about 60 kDa and a weaker band of 120 kDa. Using an immunoblot analysis (Figure 3), each of the above bands gave positive staining with a polyclonal antibody raised against barley HvCKX2 (Galuszka *et al*., unpublished results), suggesting that in the case of AtCKX7 the upper band may be an aggregated form of the protein (a dimer). Anomalously high sizes of vacuolar proteins seen on the SDS-PAGE gel

were most certainly caused by the used method (tricine-SDS-PAGE). Theoretical molecular masses for AtCKX1 and AtCKX3 are 65.05 and 59.72 kDa, respectively. This was proved by SDS-PAGE according to Laemmli (1970) as well as by MALDI-TOF intact protein mass measurement (64290.1 Da for AtCKX1, 58080.4 Da for AtCKX3 and 57057.3 Da for AtCKX7).

Table 1. Purification of recombinant vacuolar AtCKXs. Activities were determined for 250 μM iPR, in McIlvaine buffer of pH 5.0 and with 500 μM Q_0.

Purification step	Volume (ml)	Protein content (mg/ml)	Specific activity (pkat/mg)	Purification (fold)	Yield (%)
AtCKX1					
Crude extract	8700	0.03	38	1.00	100
Ultrafiltration	76	2.5	47	1.2	95.7
Octyl Sepharose	3.2	0.98	60	1.6	20.2
Hydroxyapatite	3.3	0.65	424	11	9.7
Ni-Sepharose	1.4	0.96	539	14	7.7
HEMA-BIO Phenyl	0.8	0.096	1583	41.7	1.3
AtCKX3					
Crude extract	8000	0.05	89	1.00	100.0
Ultrafiltration	62	2	167	1.87	57.9
Hydroxyapatite	3	10.2	213	2.39	18.3
Ni-Sepharose	1.1	12.1	218	2.45	8.1
Resource Q	0.25	0.46	1607	18.01	0.5

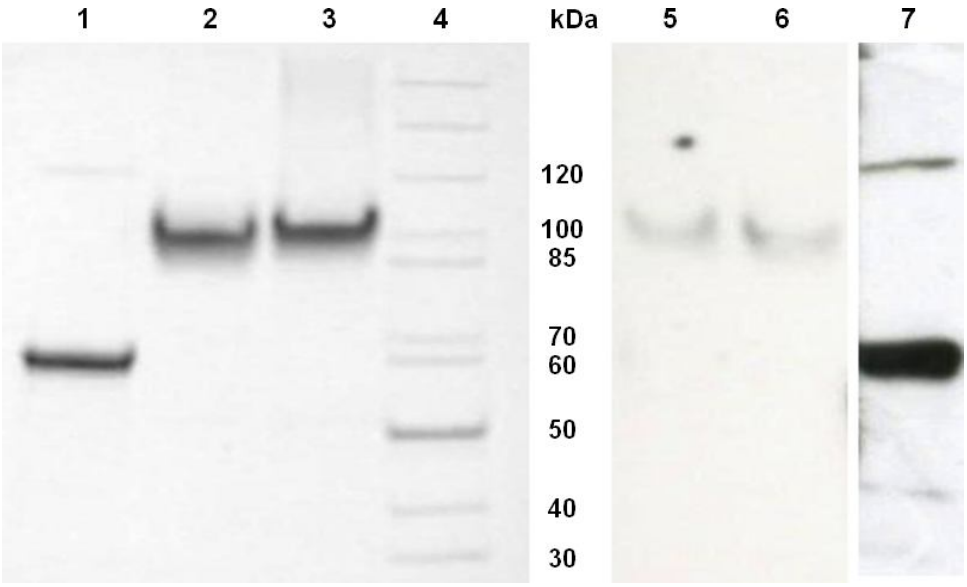
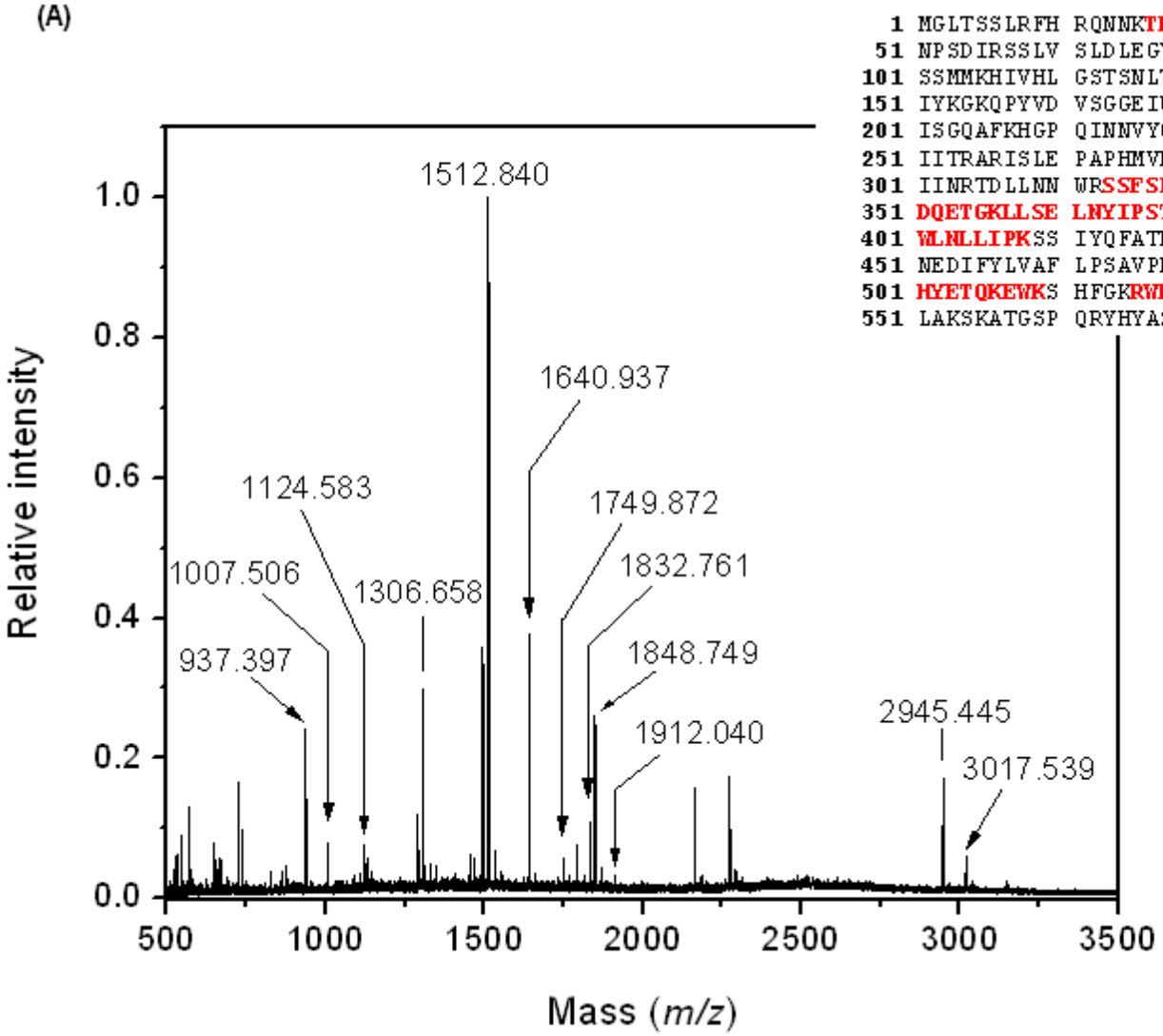


Figure 3. SDS-PAGE and immunoblotting of non-secreted AtCKX proteins. After the purification, AtCKX proteins (6 μg each) were subjected to Tricine SDS-PAGE (8% polyacrylamide gel) and either stained by Coomassie Brilliant Blue (lanes 1 to 3) or electroblotted onto PVDF membrane and detected using the polyclonal antibody against the *C*-terminal fragment of barley HvCKX2 by chemiluminescent visualisation (lanes 5 to 7). Lane description: 1 and 7 - AtCKX7, 2 and 6 - AtCKX3, 3 and 5 - AtCKX1, and 4 - molecular mass marker.

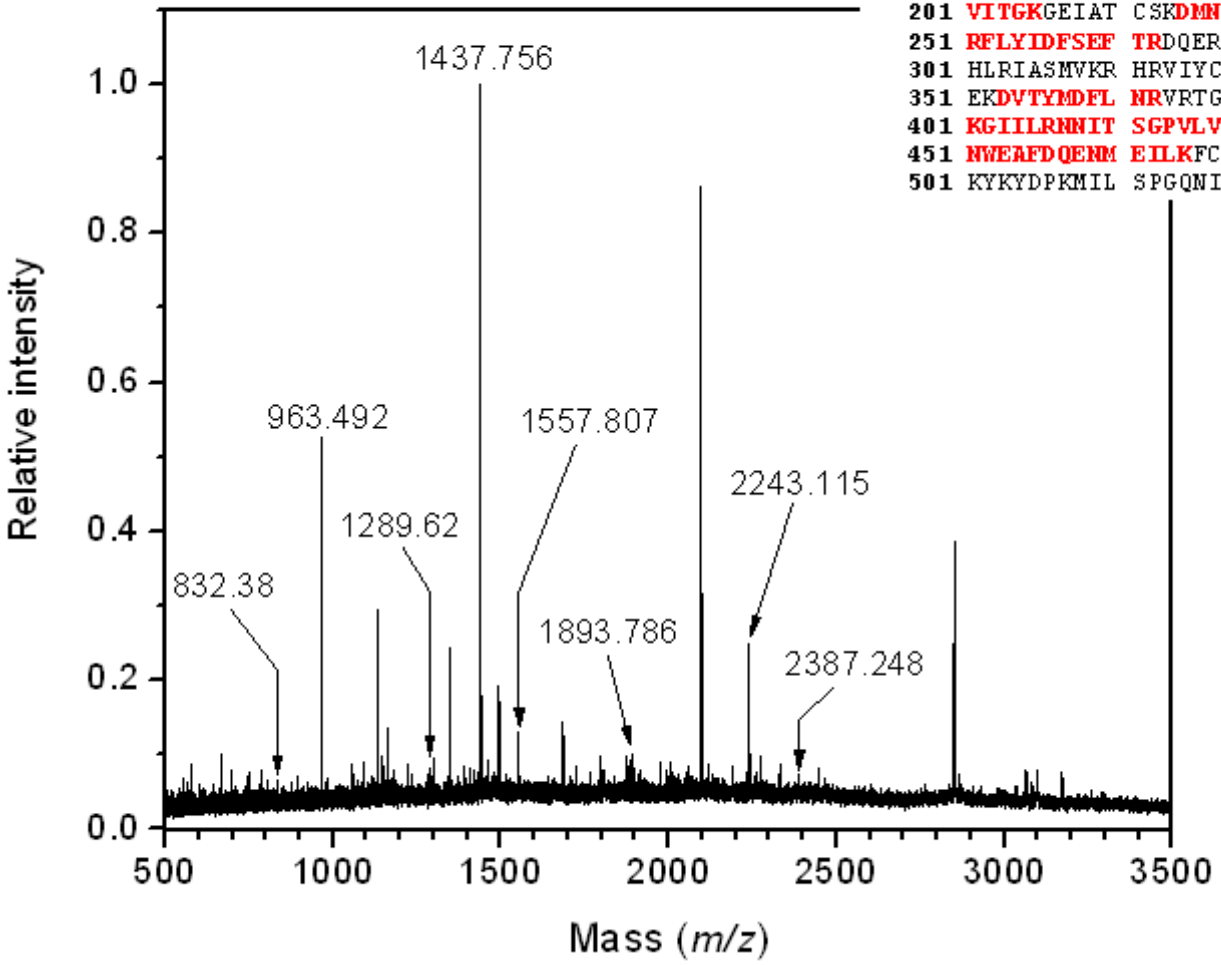
The identity of each of the recombinant and purified enzymes was confirmed using MALDI-TOF peptide mass fingerprinting after in-gel digestion (Figure 4). Recombinant AtCKX1 was unambiguously assigned to *A. thaliana* cytokinin oxidase/dehydrogenase 1 in the NCBInr database (accession no. gi|15227374; 19 matched peptides; MOWSE score: 120; sequence coverage: 30%). Recombinant AtCKX7 was unambiguously assigned to *A. thaliana* cytokinin oxidase/dehydrogenase 7 in the NCBInr database (accession no. gi|30688201; 22 matched peptides; MOWSE score: 224; sequence coverage: 46%). Recombinant AtCKX3 was unambiguously assigned to *A. thaliana* cytokinin oxidase/dehydrogenase 3 in the NCBInr database (accession no. gi|15241997; 17 matched peptides; MOWSE score: 146; sequence coverage: 35%). For clarity, tryptic peptides with *m/z* 943 and 1437 were subjected to a MALDI post-source decay analysis, which yielded sequences WNIFVER and FLYIDFSEFTR, respectively, upon reading b- and y-ion fragment series, both in accordance with the primary structure of AtCKX3 (Figure 5, Table 2).

(A)



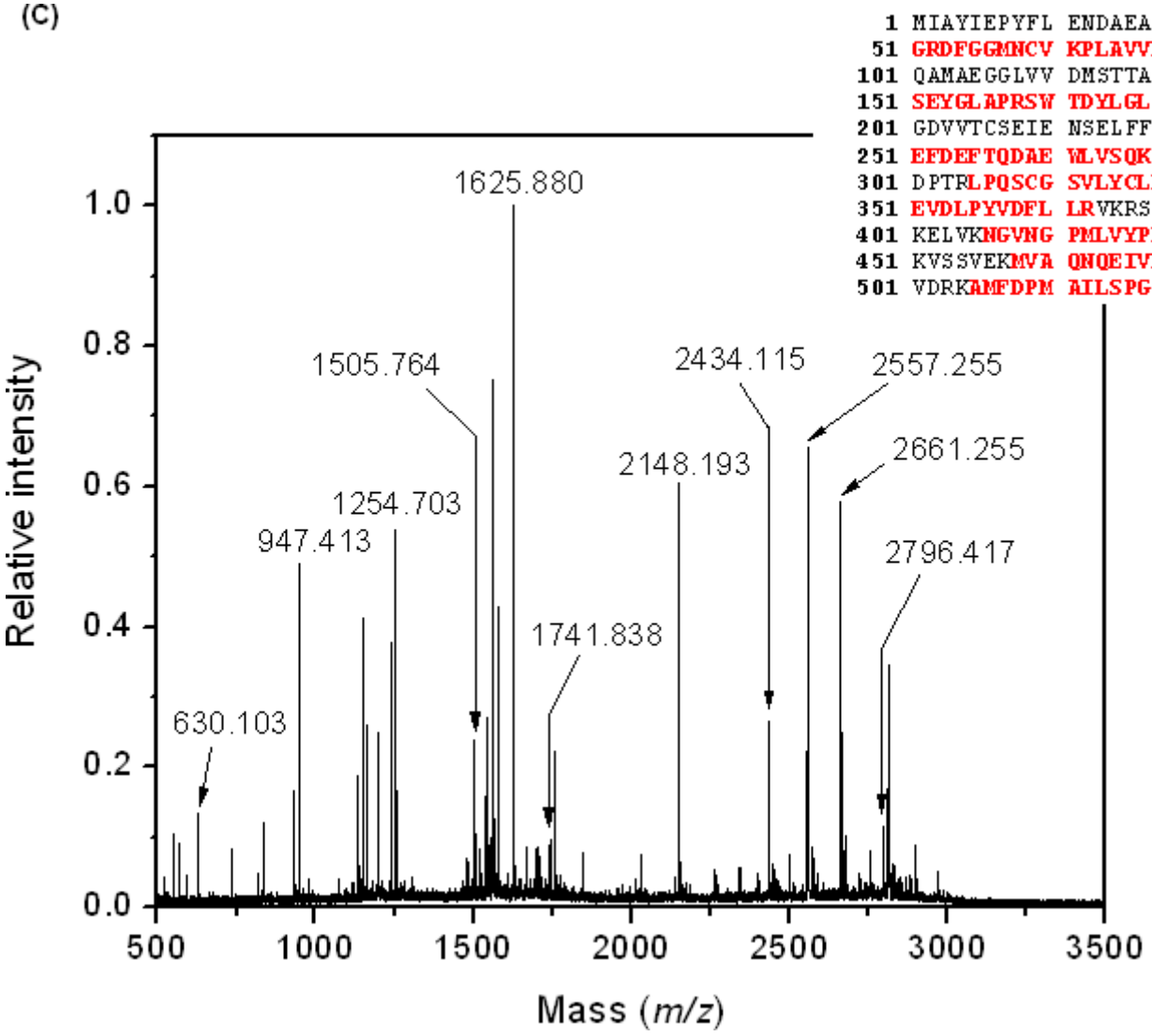

```
  1 MGLTSSLRFH RQNNKTFLGI FMILVLSCIP GRTNLCSNHS VSTPKELPSS
 51 NPSDIRSSLV SLDLEGYISF DDVHNVAKDF GNRYQLPPLA ILHPRSVFDI
101 SSMMKHIVHL GSTSNLTVAA RGHGHSLQGQ ALAHQGVVIK MESLRSPDIR
151 IYKGKQPYVD VSGGEIWINI LRETLKYGLS PKSWTDYLHL TVGGTLSNAG
201 ISGQAFKHGP QINNVYQLEI VTGKGEVVTC SEKRNSELFF SVLGGLGQFG
251 IITRARISLE PAPHMVKWIR VLYSDFSAFS RDQEYLISKE KTFDYVEGFV
301 IINRTDLLNN WRSSFSPNDS TQASRFKSDG KTLYCLEVVK YFNPEEASSM
351 DQETGKLLSE LNYIPSTLFS SEVPYIEFLD RVHIAERKLR AKGLWEVPHP
401 WLNLLIPKSS IYQFATEVFN NILTSNNNGP ILIYPVNQSK WKKHTSLITP
451 NEDIFYLVAF LPSAVPNSSG KNDLEYLLKQ NQRVMNFCAA ANLNVKQYLP
501 HYETQKEWKS HFGKRWETFA QRKQAYDPLA ILAPGQRIFQ KTTGKLSPIQ
551 LAKSKATGSP QRYHYASILP KPRTV
```

(B)




```
  1 MASYNLRSQV RLIAITIVII ITLSTPITTN TSPQPWNILS HNEFAGKLTS
 51 SSSSVESAAT DFGHVTKIFP SAVLIPSSVE DITDLIKLSF DSQLSFPLAA
101 RGHGHSHRGQ ASAKDGVVVN MRSMVNRDRG IKVSRTCLYV DVDAAWLWIE
151 VLNKTLELGL TPVSWTDYLY LTVGGTLSNG GISGQTFRYG PQITNVLEMD
201 VITGKGEIAT CSKDMNSDLF FAVLGGLGQF GIITRARIKL EVAPKRAKWL
251 RFLYIDFSEF TRDQERVISK TDGVDFLEGS IMVDHGPPDN WRSTYYPPSD
301 HLRIASMVKR HRVIYCLEVV KYYDETSQYT VNEEMEELSD SLNHVRGFMY
351 EKDVTYMDFL NRVRTGELNL KSKGQWDVPH PWLNLFVPKT QISKFDDGVF
401 KGIILRNNIT SGPVLVYPMN RNKWNDRMSA AIPEEDVFYA VGFLRSAGFD
451 NWEAFDQENM EILKFCEDAN MGVIQYLPYH SSQEGWVRHF GPRWNIFVER
501 KYKYDPKMIL SPGQNIFQKI NSS
```

(C)




```
  1 MIAYIEPYFL ENDAEAASAA TAAGKSTDGV SESLNIQGEI LCGGAAADIA
 51 GRDFGGMNCV KPLAVVRPVG PEDIAGAVKA ALRSDKLTVA ARGNGHSING
101 QAMAEGGLVV DMSTTAENHF EVGYLSGGDA TAFVDVSGGA LWEDVLKRCV
151 SEYGLAPRSW TDYLGLTVGG TLSNAGVSGQ AFRYGPQTSN VTELDVVTGN
201 GDVVTCSEIE NSELFFSVLG GLGQFGIITR ARVLLQPAPD MVRWIRVVYT
251 EFDEFTQDAE WLVSQKNESS FDYVEGFVFV NGADPVNGWP TVPLHPDHEF
301 DPTRLPQSCG SVLYCLELGL HYRDSDSNST IDKRVERLIG RLRFNEGLRF
351 EVDLPYVDFL LRVKRSEEIA KENGTWETPH PWLNLFVSKR DIGDFNRTVF
401 KELVKNGVNG PMLVYPLLRS RWDDRTSVVI PEEGEIFYIV ALLRFVPPCA
451 KVSSVEKMVA QNQEIVHWCV KNGIDYKLYL PHYKSQEEWI RHFGNRWSRF
501 VDRKAMFDPM AILSPGQKIF NRSL
```

Figure 4. MALDI-TOF peptide mass fingerprinting of (A) AtCKX1 (B) AtCKX3 and (C) AtCKX7. MALDI-TOF mass spectra of tryptic digests were acquired using an α-cyano-4-hydroxycinnamic acid matrix. Matching peptides are shown in bold in each sequence (see the Experimental section for details).

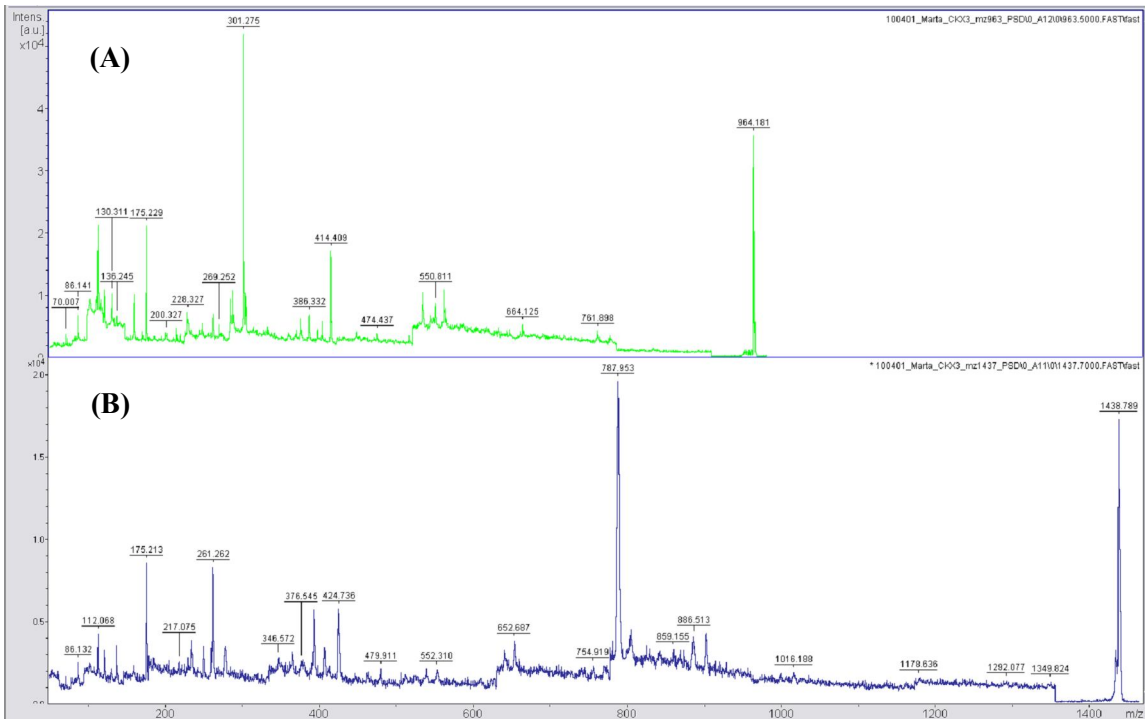


Figure 5. MALDI post-source decay analysis of AtCKX3 tryptic peptides with *m/z* 943 (A) and 1437 (B).

Table 2. MALDI post-source decay analysis of AtCKX3 tryptic peptides with *m/z* 943 and 1437, respectively.

N-Term.	Ion	a	b	y	i	a-17	b-17	C-Term.	Ion
1	W	159.092	187.087	175.119	159.092	142.065	170.060	7	R
2	N	273.135	301.130	304.162	87.055	256.108	284.103	6	E
3	I	386.219	414.214	403.230	86.096	396.192	397.187	5	V
4	F	533.287	561.282	550.298	120.080	516.261	544.255	4	F
5	V	632.355	660.350	663.382	72.080	615.329	643.324	3	I
6	E	761.398	789.393	777.425	102.054	744.372	772.366	2	N
7	R	917.499	945.494	963.505	129.113	900.473	928.468	1	W

N-Term.	Ion	a	b	y	i	a-17	b-17	C-Term.	Ion
1	F	120.081	148.076	175.119	120.081	103.054	131.049	11	R
2	L	233.165	261.160	276.167	86.096	216.138	244.133	10	T
3	Y	396.228	424.223	423.235	136.075	379.202	407.197	9	F
4	I	509.312	537.307	552.278	86.096	492.286	520.281	8	E
5	D	624.339	652.334	639.310	88.039	607.313	635.308	7	S
6	F	771.408	799.403	786.378	120.080	754.381	782.376	6	F
7	S	858.440	886.435	901.405	60.044	841.413	869.408	5	D
8	E	987.482	1015.477	1014.489	102.054	970.456	998.451	4	I
9	F	1134.551	1162.546	1177.552	120.080	1117.524	1145.519	3	Y
10	T	1235.598	1263.593	1290.636	74.059	1218.572	1246.567	2	L
11	R	1391.699	1419.694	1437.705	129.113	1374.673	1402.668	1	F

Properties of non-secreted AtCKX enzymes

It is likely that each cellular compartment will contain different cytokinins and different cytokinin conjugates and so there is an interest to investigate how substrate specificities vary between each of the CKX isoforms. Consequently, purified recombinant enzymes were characterized for their preference in both electron acceptor and substrate using a modified end-point method (Frébort *et al*., 2002). Specific activities of each enzyme were measured with iPR as a substrate at both pH 5.0 and pH 7.5 using a set of electron acceptors. The basal level of enzyme activity using molecular oxygen as an acceptor was used as a reference (Galuszka *et al*., 2001; Frébortová *et al*., 2004).

Table 3. CKX specificity for the electron acceptor. Recombinant non-secreted AtCKX proteins were compared in their CKX activities in the presence of 500 μM electron acceptor in McIlvaine buffer pH 5.0 and 7.5 with 250 μM iPR as a substrate. The values show ratio of CKX activities to the activity obtained without externally added electron acceptor (with O_2).

Electron acceptor	Relative CKX activities					
	AtCKX1		AtCKX3		AtCKX7	
	pH 5.0	pH 7.5	pH 5.0	pH 7.5	pH 5.0	pH 7.5
Oxygen	1	1	1	1	1	1
3',5'-Dimethoxy-4-hydroxyacetophenone	0.7	0.4	0.3	0.2	1.1	1.3
Cytochrome *c*	0.8	0.3	1.9	0.9	1.5	2.1
2,3,5-Triphenyl-tetrazolium chloride	4	4	1	0.9	1.1	1.6
Ferricyanide	552	130	7.2	1.3	59.5	2.9
NAD^+	1.5	1.4	0.8	0.8	1.2	1.5
2,6-Dichlorophenol indophenol	25	49	0.6	0.2	2.6	15.4
2,3-Dimetoxy-5-methyl-1,4-benzoquinone (Q_0)	117	238	3.2	2.7	19.9	5.6

In slightly acidic conditions, ferricyanide was found to be the prefered electron acceptor for all three enzymes, enhancing enzymatic activity 552-fold, 7.2-fold and 59.5-fold for AtCKX1, AtCKX3 and AtCKX7, respectively (Table 3). At neutral pH, AtCKX7 prefered DCPIP as the electron acceptor (15.4-fold higher activity), whereas vacuolar AtCKX enzymes gave higher reaction rates with Q_0 (238-fold for AtCKX1 and 2.7-fold for AtCKX3, respectively). It is noticeable that the activity of AtCKX3 was only modestly enhanced over the activity with oxygen, suggesting that this enzyme is either a less effective dehydrogenase compared to other AtCKXs, or prefers a different, but unknown electron acceptor.

Having established that all enzymes performed well with Q_0 as electron acceptor, substrate specificities were assessed in 500 μM Q_0. For ease of comparison, data are presented relative to the specific activity with iP as substrate. Both AtCKX1 and AtCKX7 showed considerable preference for certain conjugates over iP. Interestingly, iP9G was the best substrate for AtCKX7 and one of the best substrates for AtCKX1 (approx 40-fold increase in activity above iP in each case), although being a poor substrate for AtCKX3 (Figure 6). AtCKX1 showed highest preference for tZ phosphates (up to 96-fold elevation of activity for tZTP over iP) and also effectively degraded iP phosphates (up to 30-fold for iPTP). Surprisingly, AtCKX3 did not demonstrate strong preference for any studied cytokinin, even though preferred iP phosphates and iPR over iP (less than 3-fold).

AtCKX7 also very efficiently degraded tZ and iPR (20-fold and 4.5-fold, respectively). Similar preference for tZ as well as for iP9G was previously observed for cytosolic ZmCKX10 (Šmehilová *et al*., 2009), while apoplastic ZmCKX1 and AtCKX2 degrade tZ most efficiently and have very low activity with iP9G and iPMP (Bilyeu *et al*., 2001; Šmehilová *et al*., 2009; Frébortová *et al*., 2007; Galuszka *et al*., 2007). None of the intracellular AtCKX enzymes showed high activity with aromatic cytokinins. AtCKX7 was able to degrade kinetin at 28% of the rate for iP and AtCKX1 was able to degrade kinetin riboside at 50% of the rate for iP. No activity was recorded against kinetin or its riboside for AtCKX3. Activities using oxygen as the electron acceptor were generally much lower (Table 3), but substrate preferences were essentially unchanged (Figure 6b).

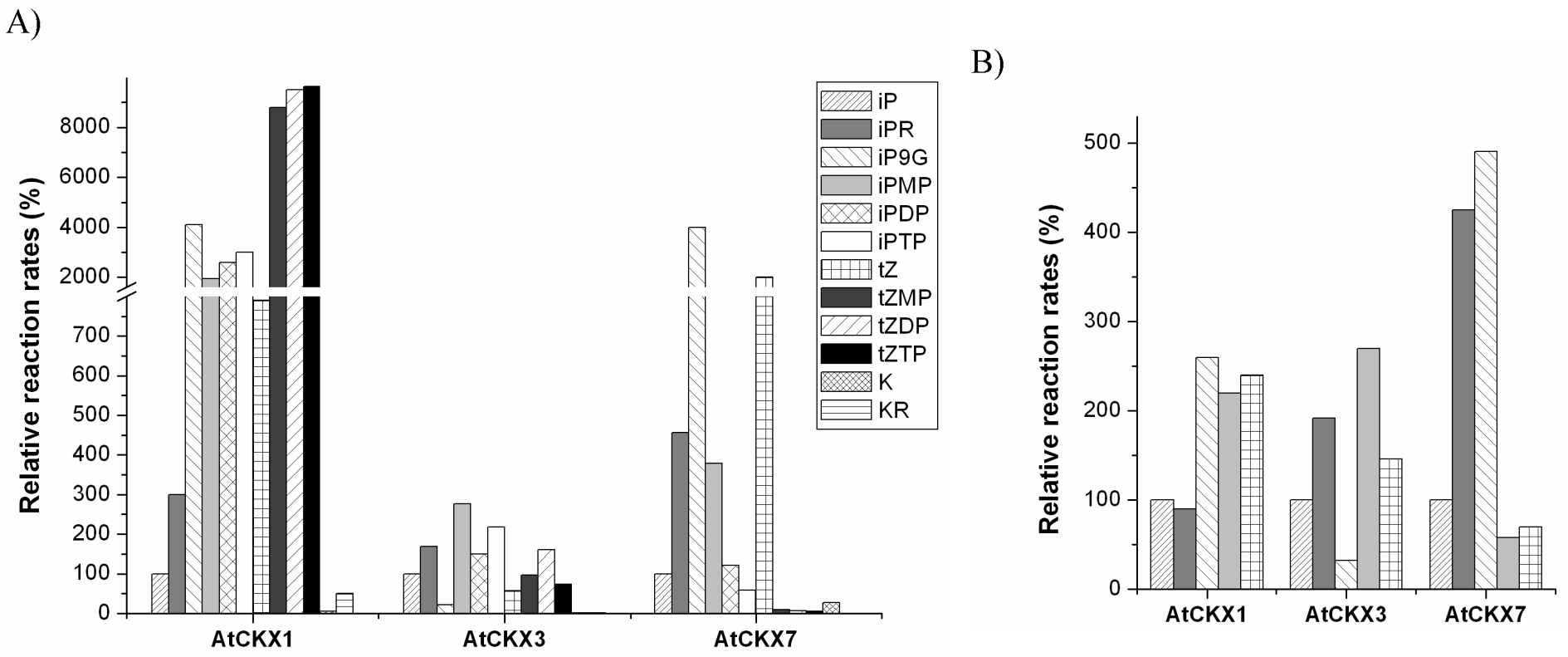


Figure 6. Comparison of substrate specificities of non-secreted AtCKX proteins given as relative reaction rates towards iP (100%). Measurements were done in McIlvaine buffer (pH 5.5) with 200 μM substrates and either 500 μM electron acceptor Q_0 (2,3-dimetoxy-5-methyl-1,4-benzoquinone) for dehydrogenase reaction (A) or no electron acceptor added for oxidase reaction (B). Specific activity with iP was 266 pkat/mg for AtCKX1, 416 pkat/mg for AtCKX3 and 1776 pkat/mg for AtCKX7.

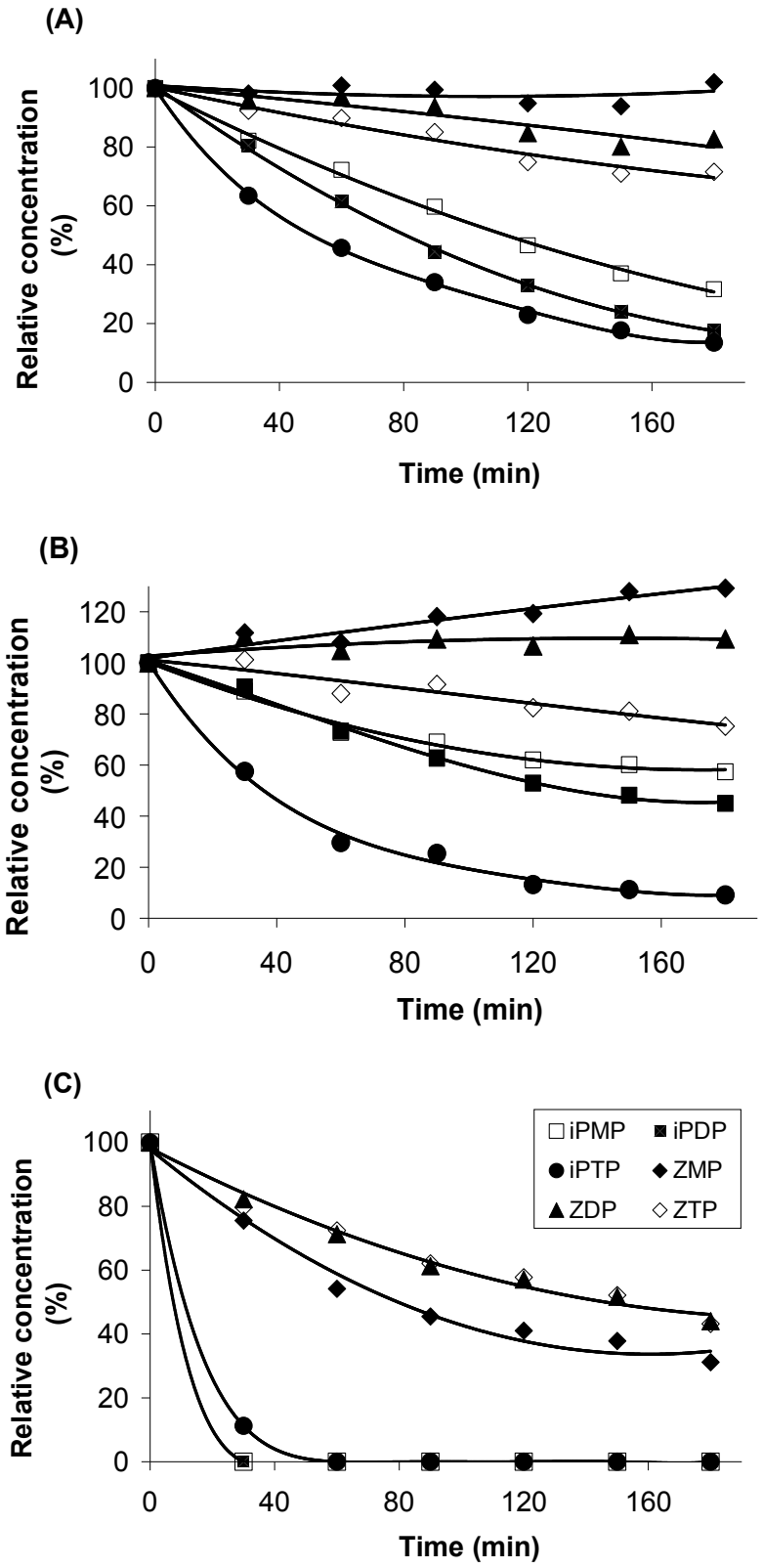


Figure 7. Depletion of cytokinin phosphates from dehydrogenase reaction of (A) AtCKX1 (11 μg), (B) AtCKX3 (4 μg) and (C) AtCKX7 (12 μg). The decrease in concentration of initial 100 μM solution of each cytokinin in 50 mM MES/Tris buffer pH 5.0 in the presence of 500 μM Q_0 was followed by capillary electrophoresis. cZ and tZ phosphates were impossible to separate.

Reaction of vacuolar and cytoplasmic AtCKX enzymes with cytokinin nucleotides was further studied by incubating the purified enzymes in a mixture of nine substrates and analyzing residual cytokinins by capillary electrophoresis (Figure 7). In general, iP phosphates were better substrates than zeatin phosphates for all AtCKX enzymes studied (*cis*- and *trans*-zeatin phosphates were impossible to separate due to their migration as one peak). Vacuolar AtCKX enzymes preferentially degraded tri- and then diphosphates over monophosphates of isopentenyladenine. Insignificant degradation of zeatin monophosphates by either AtCKX1 or AtCKX3 was observed after the 3-h incubation, in contrast to tri- and diphosphates which rapidly disappeared from the mix, especially in the case of AtCKX1 (Figure 7a, b). The cytosolic enzyme AtCKX7 prefered zeatin monophosphate over di- and triphosphates, however these activities were trivial compared to those for iP phosphates (Figure 7c). Under the same conditions, relative reaction rates for AtCKX1, AtCKX3 and AtCKX7 against iPMP vs. iP were 13, 10 and 1, respectively. In contrast, the secreted enzymes AtCKX2 and ZmCKX1 had reaction rates for iPMP lower by two orders of magnitude than for iP and the degradation of di- and triphosphates was even less favoured, close to the detection limit of the assay method (data not shown).

Concluding remarks

Amino acid sequence analysis of both AtCKX1 and CKX3 revealed that they contain degenerate *N*-terminal sequence-specific vacuolar sorting signal (ssVSS) consisting of 4 amino acids known as the NPIR consensus. For the first time recombinant vacuolar and cytoplasmic cytokinin dehydrogenase enzymes from *A. thaliana* were obtained and characterised. Their preferences towards different electron acceptors and preferences towards different cytokinins and cytokinin nucleotides were determined. It was found that the activity of vacuolar AtCKX1 protein strongly depends on the presence of an appropriate electron acceptor. Vacuolar AtCKX3 and cytosolic AtCKX7 showed less preference for electron acceptor, but remained more efficient as dehydrogenases than as oxidases (Table 2). In contrast to the substrate specificities recorded for secreted CKXs, all three non-secreted AtCKX proteins were able to cleave all types of endogenous cytokinin nucleotides. It is interesting to consider on the preference of AtCKX1 and AtCKX7 isoforms for cytokinin *N*-glucoside and phosphates over the preference of secreted CKXs. This is likely to be a reflection of the prevalence of particular cytokinin derivatives within each intracellular compartment and illustrates that cytokinin concentrations are actively managed throughout the cell as well as throughout the plant.

When AtCKX genes were constitutively overexpressed in *Arabidopsis* or tobacco plants, all had an adverse impact on the phenotype, although diverse cytokinin deficiencies were seen (Werner *et al*., 2001, 2003). Overproduction of AtCKX1 and AtCKX3 caused the most severe phenotype with

dwarfed aerial, vegetative tissues and very low fertility. Clearly the vacuolar pool of cytokinin nucleotides and glucosides is critical. Depletion by vacuolar CKXs appears to act as a net sink of cytokinins suggesting that the vacuolar pool could be actively managed. Strict compartmentation of cytokinin biosynthesis to plastids and cytokinin degradation to other major compartments is understandable, although the need for multiple sites for degradation complicates the picture. The full physiological significance of the high ability of vacuolar and cytoplasmic CKXs to cleave cytokinin metabolites remains so far unclear. Elucidation of the role of unique cytosolic CKX isoenzyme in cytokinin homeostasis is a challenging task considering its substrate specificity and almost constitutive and abundant pattern of expression (Šmehilová *et al*., 2009; Vyroubalová *et al*., 2009).

The k_{cat} values for each of the AtCKX enzymes has been collated from this dataset and published data for apoplastic AtCKX2 and ZmCKX1 and cytosolic ZmCKX10 (Table 4). Vacuolar AtCKX3 showed very low k_{cat} compared to other enzymes, even though all the values were determined under the best known conditions. However it is possible that an alternative electron acceptor or cytokinin is preferred. For example, a recent finding that ZmCKX1 has very high k_{cat} in the presence of ABTS radical (Frébortová *et al*., 2010) made us wonder if an unknown, vacuole-specific electron acceptor might exist for these enzymes. It is known that radicals can be found in vacuoles, for example the oxidation of phenolic components in peroxidase-dependent reactions produces phenoxyl radicals, which are then reduced by ascorbic acid to form other radicals as intermediates (Takahama *et al*., 2004). It is clear that there is still more to learn about cytokinin homeostasis.

Table 4. Comparison of highest reported k_{cat} values of most studied AtCKX and ZmCKX proteins.

Enzyme	Substrate	Electron acceptor	pH	k_{cat} (1/s)	Reference
AtCKX1	250 μM tZTP	500 μM ferricyanide	5.0	21.4	this work
AtCKX2	150 μM tZ	500 μM Q_0	7.0	13.6	calculated from Frébortová *et al*., 2007
AtCKX3	250 μM iPR	500 μM ferricyanide	5.0	1.2	this work
AtCKX7	250 μM iP9G	500 μM ferricyanide	5.0	17.9	this work
ZmCKX1	150 μM iP	500 μM Q_0	6.5	143.6	Frébortová *et al*., 2004
ZmCKX1	150 μM iP	100 μM ABTS radical	5.0	258.7	calculated from Frébortová *et al*., 2010
ZmCKX10	50 μM tZ	250 μM ferricyanide	6.5	0.84	calculated from Šmehilová *et al*., 2009

Experimental

Plant material

Transgenic tobacco plants overexpressing *AtCKX1* (At2g41510) and *AtCKX3* (At5g56970) genes from *A. thaliana* were prepared as reported previously (Werner *et al*., 2001). Tobacco *AtCKX7* (At5g21482) overexpressor was prepared by the same procedure (Werner *et al*., unpublished). Plants were cultured in a greenhouse with 15-h light / 9-h dark cycles, at 25 °C. Leaf material was harvested before flower induction, frozen immediately in liquid nitrogen and stored at -80 °C.

Isolation of AtCKX genes

The starting material for RNA isolation were leaves of transgenic tobacco plants overexpressing the genes of interest (Werner *et al*., 2001). RNA was isolated with Plant RNA 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 for each gene were designed so that resulting amplicons would be missing predicted *N*-terminal signal sequences (SignalP 3.0 Server, Bendtsen *et al*., 2004; Fig. 1). Genes were amplified with the use of Phusion DNA Polymerase (Finnzymes, Espoo, Finland). A TGradient Thermocycler (Biometra, Goettingen, Germany) was programmed as follows: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 60 s at 60/65/60 °C (AtCKX1, AtCKX3 and AtCKX7, respectively), 30 s at 72 °C; and terminated by 10 min at 72 °C. Genes encoding vacuolar enzymes were further cloned into pGAPZα shuttle vector (Invitrogen), containing the *S. cerevisiae* α-factor secretion signal, and plasmid constructs were transformed into *E. coli* TOP10F (Invitrogen) by electroporation at 1.8 kV. Selection of transformants was based on zeocin resistance. *AtCKX7* was cloned into pPICZA shuttle vector (Invitrogen). All primer sequences are shown in Table 5.

Preparation of pGAPZα vector with an additional His-tag before multiple cloning site

To prepare pGAPZα vector containing an additional polyhistidine domain, the pET16b vector (Novagen, Madison, WI, USA) was used. The pET16b vector carries an *N*-terminal His-Tag sequence and this was precloned into pGAPZα . The resulting pGAPZαA$(His)_{10}$ vector was used as an expression vector for vacuolar AtCKX proteins in *P. pastoris* X-33.

Table 5. Primer sequences used for cloning of AtCKXs genes into *Pichia* transformation vector.

Gene	Vector	Primer sequence 5´ to 3´-end direction	Restriction site
CKX1	pGAPZαA	gcctcgagTGTTCCAATCATTCTGTTAGTA	*XhoI*
		gccgcggTTATACAGTTCTAGGTTTCGG	*SacII*
	pGAPZαA(His)$_{10}$	tccccgcggGTTCCAATCATTCTGTTAG	*SacII*
		acgcgtcgacTTATACAGTTCTAGG	*SalI*
CKX3	pGAPZαC	ccatcgattTTCACACAACGAATTCG	*ClaI*
		cggggtaccCTAACTCGAGTTTATTTTTTGA	*Asp718*
	pGAPZαA(His)$_{10}$	gtccatatgTCACACAACGAATTCGC	*NdeI*
		cggggtaccCTAACTCGAGTTTATTTTTTGA	*Asp718*
CKX7	pPICZA	gcggtaccATGATAGCTTACATAGAACCATACT	*Asp718*
		tccccgcggCAAAGAGACCTATTGAAAATC	*SacII*

Production of recombinant AtCKX enzymes in Pichia pastoris

The plasmid constructs pGAPZα::AtCKX were linearized with *AvrII* (NEB, Ipswich, MA, USA) and used for integration into the *P. pastoris* X-33 (Invitrogen) genome. Yeast transformation was done by electroporation at 1.5 kV according to the manufacturer's protocol. The transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% D-glucose, 1 M sorbitol, 2% agar) containing 100 mg/l zeocin (Duchefa Biochemie B.V., Haarlem, The Netherlands). Selected transformants were picked up and grown for one day in 2 ml of YPD medium (1% yeast extract, 2% peptone, 2% D-glucose) with 100 mg/l zeocin, at 30 ºC with extensive shaking at 230 rpm. The pGAPZα::AtCKX transformants were then transferred into 50 ml of YPD medium without zeocin buffered to pH 7.2 with 0.1 M potassium phosphate buffer and cultivated for 48 h at 28 ºC with 230 rpm shaking. After that yeast cells were removed by centrifugation at 5000*g* for 10 min and the CKX activity was measured in the cell-free medium.

The pPICZA::AtCKX7 plasmid construct was linearized with *SacI* (Takara, Kyoto, Japan) and used for integration into the *P. pastoris* X-33 genome as described above. 60 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, $4 \cdot 10^{-5}$% biotin and 1% glycerol) in 250 ml flask was inoculated by an overnight culture of *P. pastoris* pPICZA::AtCKX7 (grown with 100 mg/l of zeocin) and cultivated for 16-18 h at 30 °C and 180 rpm. The cells were harvested by centrifugation, resuspended to OD_{600} of 1 in BMMY medium (1% yeast

extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, $4 \cdot 10^{-5}$% biotin and 0.5% methanol), divided to 200 ml aliquots and cultivated in 500 ml flasks at 28 °C and 230 rpm. After 22 h the culture medium was supplemented with MeOH to a final concentration of 0.5%, the cells were cultivated for next 4 h and then harvested by centrifugation. *Pichia* cells were resuspended in breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol) and disrupted by vortexing with an equal volume of acid-washed glass beads (425-600 μM, Sigma). After centrifugation activity was measured in the cell lysate.

Production of recombinant AtCKX7

The ORF of AtCKX7 was subcloned into the pTYB12 vector through *NdeI* and *EcoRI* restriction sites (NEB) and transformed into *E. coli* BL21 (DE3) STAR (Invitrogen). The transformant culture was diluted to OD_{600}=0.1, grown at 22 °C until it reached OD_{600}=0.5 and subsequently induced for 5 h at 18 °C with 0.5 mM IPTG. The cells were collected and resuspended in 60 ml of 0.02 M Tris/HCl (pH 8.0), 0.1% Triton-X 100, 500 mM NaCl and 1 mM EDTA and disrupted by a French press (20000 psi) (Thermo, Waltham, MA, USA). The lysate was centrifuged and the supernatant purified via chitin affinity chromatography. Twenty ml of chitin resin (NEB; 5 x 1.6 cm) was equilibrated in 0.02 M Tris/HCl (pH 8.0), 0.1% Triton-X 100, 500 mM NaCl and 1 mM EDTA. The sample was loaded onto the column and washed with 80 ml of equilibration buffer. Subsequently, 60 ml of equilibration buffer supplemented with 50 mM DTT was passed through the column to induce intein tag cleavage and the sample was incubated at 16 °C for 62 h. AtCKX7 protein was then eluted with 50 ml of equilibration buffer without DTT. The elution fraction was concentrated on Amicon centrifugal cellulose filter with cut off 10 kDa (Millipore) and stored at -20 °C.

Preparation of pPIC9K vector under control of constitutive GAP promoter

pPIC9K vector (Invitrogen) and proper plasmid constructs pGAPZαA$(His)_{10}$::AtCKX were subjected to partial digestion with *BglII* (Takara) and *BshTI* (Fermentas). Digestion products of expected size (approx. 8 kb for pPIC9K and 2.4-2.5 kb for pGAPZαA$(His)_{10}$::AtCKX) were further ligated together and transformed into *E. coli* TOP10F (Invitrogen) by electroporation at 1.8 kV. Selected plasmid constructs pPIC9K::AtCKX were subsequently linearized with *AvrII* (NEB) and used for integration into *P.pastoris* SMD1168 (Invitrogen) genome. His^+ transformants were generated on MD plates (1.34% yeast nitrogen base without amino acids (Difco™, Detroit, MI, USA), $4 \cdot 10^{-5}$% biotin, 2% D-glucose, 2% agar) and screened for multicopy inserts on YPD plates (1% yeast extract, 2% peptone, 2% D-glucose, 1.5% agar) containing various concentrations (from 0.5 to 3 mg/ml) of Geneticin® (G-418 sulfate) (Calbiochem, Merck, Darmstadt, Germany). Selected transformants were picked up and grown for one day in 2 ml of YPD medium with a proper concentration of Geneticin, at

30 ºC with extensive shaking at 230 rpm. The pPIC9K::AtCKX transformants were then transferred into 50 ml of YPD medium without antibiotic buffered to pH 7.2 with 0.1 M potassium phosphate buffer and cultivated for 48 h at 28 ºC with 230 rpm shaking. After that yeast cells were removed by centrifugation at 5000*g* for 10 min and the CKX activity was measured in the cell-free medium.

Purification of vacuolar AtCKX proteins

Vacuolar AtCKXs were purified from the cell free medium of *P. pastoris* SMD1168 transformed with pPIC9K::AtCKX. The yeasts were cultivated at 30 °C in a medium (200 ml) containing 13.4 g/l of yeast nitrogen base without amino acids (Difco^{TM}), 0.1 M potassium phosphate buffer (pH 7.2) and 2% D-glucose, with orbital shaking at 230 rpm. After 24-h cultivation the culture was used as an inoculum of 8 l of fermentation medium (13.4 g/l of yeast nitrogen base without amino acids, 0.1 M potassium phosphate buffer (pH 6.5) 1% glycerol and 0.3 ml of antifoam A (Sigma). Protein expression was carried out in R'ALF Plus Duet fermentor (Bioengineering AG, Wald, Switzerland) of a 10 l working volume. Process temperature was maintained at 30 °C and pH was controlled by the addition of 5 M KOH. Foam formation was reduced with antifoam A. Fed-batch fermentation was started after about 40 h, depending on the oxygen level. The appearance of dissolved oxygen spike indicated the depletion of the initial glycerol and the need for feeding. The fed-batch medium consisted of (per liter of deionized water): 500 g D-glucose, 2.4 mg D-biotin and 4 ml trace salts solution (per liter of deionized water: H_3BO_3 0.02 g, $CuSO_4 \cdot 5H_2O$ 2 g, KI 0.1 g, $MnSO_4 \cdot H_2O$ 3 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, $ZnSO_4 \cdot 7H_2O$ 17.8 g, $CoCl_2$ 0.92 g) and it was fed at a rate of 0.2 ml/min. Samples were taken in different time points to monitor CKX activity and culture density over time. After about 50 h of feeding fermentation process was stopped and yeast cells were removed by centrifugation at 4600*g*. The cell-free medium was concentrated to about 60 ml by ultrafiltration on a MiniKros Sampler Module (Spectrum, Rancho Dominguez, CA, USA) with the cut-off 10 kDa. Ultrafiltration was repeated three times to substitute buffered media for 50 mM Tris/HCl (pH 8.0) supplemented with 20% ammonium sulphate or 10 mM potassium phosphate buffer (pH 7.4) in the case of AtCKX3. The concentrated AtCKX1 proteins were loaded on an Octyl Sepharose 4 Fast Flow hydrophobic column (GE Healthcare; 18 x 2.8 cm) connected to BioLogic LP chromatograph equipped with UV and conductivity detector (Bio-Rad). After applying the sample, the column was washed with a descending linear gradient of ammonium sulfate and eluate was fractionated. The fractions showing enzyme activity were pooled and concentrated using a stirred ultrafiltration cell (Millipore, Bedford, MA, USA) equipped with a *YM 10* membrane (cut-off 10 kDa). The concentrated protein sample was applied onto a Bio-Gel Hydroxyapatite (Bio-Rad; 18 x 2.8 cm). The column was equilibrated with 10 mM potassium phosphate buffer (pH 7.6) and the proteins were then eluted by a linear gradient of 10 mM and 500 mM potassium phosphate buffers (pH 7.6, 200 ml, 0-50%), followed by a short linear gradient to 100% of 500 mM potassium phosphate buffer (pH 7.6, 100 ml)

and an isocratic elution with the same buffer (100 ml). Active fractions were concentrated to 2 ml and the buffer was exchanged for 50 mM potassium phosphate (pH 7.4) containing 0.5 M NaCl using the ultrafiltration device with 30 kDa membrane cut-off (Millipore). CKX samples were finally purified on a Ni Sepharose HP (GE Healthcare; 9.5 x 1 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 M NaCl. His-tagged proteins were washed from the column by imidazole added to the starting buffer in the range of 10 to 50 mM. Active fractions were collected, concentrated by ultrafiltration and the buffer was exchanged to 50 mM Tris/HCl (pH 8.0) supplemented with 20% ammonium sulfate or 20 mM Bis-Tris/HCl (pH 5.0) for AtCKX1 and AtCKX3, respectively. The AtCKX1 sample was loaded onto HEMA-BIO 1000 Phenyl column (Tessek, Prague, Czech Republic; 7.5 x 70 mm) that was subsequently washed with descended linear gradient of ammonium sulfate and eluate was fractionated. The AtCKX3 sample was loaded onto Resource Q column (GE Healthcare; 6 ml) and washed out by increasing concentration of NaCl in the 20 mM Bis-Tris/HCl buffer. Both columns were connected to BioLogic Duo-Flow FPLC system equipped with UV and conductivity detector (Bio-Rad). Fractions with CKX activity were pooled, concentrated by ultrafiltration and stored at -20 °C.

Protein content in enzyme samples was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

SDS-PAGE and immunoblot analysis

Tricine-SDS-PAGE was performed on a slab gel (8%) in Tris-tricine running buffer according to Schägger (1987). Recombinant molecular weight standard mixture (Sigma) was used as a marker. Protein samples were heated before application at 100 °C for 10 min in the presence of 1% SDS and 1% 2-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany) or proteins were blotted onto PVDF membrane (0.45 μm) in the MiniTrans blot system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% powdered milk in 20 mM Tris/HCl, (TBS buffer, pH 7.6) for at least 1 h, washed with TBS containing 0.1% of Tween-20 (Tween-TBS buffer) and incubated for at least 1 h in TBS containing 1% powdered milk and a rabbit polyclonal antibody raised against the *C*-terminal fragment of barley HvCKX1 or HvCKX2 (Galuszka *et al*., unpublished results). The membrane was subsequently washed four times with Tween-TBS and incubated for 1 h with anti-rabbit IgG horseradish peroxidase conjugate (Sigma) in TBS containing 1% powdered milk. After rinsing in Tween-TBS buffer, membranes were developed with Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK) and Lumi-film chemiluminescent detection film (Roche, Basel, Switzerland) according to the manual.

Identification of recombinant proteins by MALDI-TOF mass spectrometry

Protein bands (containing picomolar protein amounts) were excised from Coomassie-stained SDS-PAGE gels. MALDI-TOF peptide mass fingerprinting was conducted after a previous in-gel digestion of samples by modified trypsin (Šebela *et al*., 2006). The digestion protocol additionally involved reduction and alkylation steps (Shevchenko *et al*., 2007) and proceeded overnight at 37 °C. The instrument used was a Microflex LRF20 MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a microScout ion source and a 337-nm nitrogen laser (10 Hz). Measurements were performed in the reflectron mode for positive ions. Parameters of the instrument were as follows: an acceleration voltage of 19 kV, an extraction voltage of 16.1 kV, a lens voltage of 9.1 kV, a reflectron voltage of 20 kV and a delayed extraction of 250 ns. Digest aliquots (0.6 µl) were pipetted onto an MSP AnchorChip^{TM} 600/96 target plate, overlaid with 0.6 µl of a matrix solution (5 mg α-cyano-4-hydroxycinnamic acid in 0.33 ml of 2.5% v/v trifluoroacetic acid and 0.66 ml acetonitrile) and left to dry in air. Mass spectra were accumulated from 100–200 laser shots, the examined *m/z* range was 500-6000; a mixture of peptide standards (Bruker Daltonik) was used for external calibration. The acquired spectra were processed by flexAnalysis 2.4 and Biotools 3.2 software (Bruker Daltonik). Database searches were performed against Swiss-Prot (release 57.12) and NCBInr (release December 15, 2009) databases using the program Mascot Server 2.2 (Matrix Science, London, UK). As variables, oxidation of methionine and carbamidomethylation of cysteine plus one missed cleavage were chosen for all searches performed without taxonomic restriction; a mass tolerance of 150 ppm was allowed. Post-source decay (PSD) spectra of selected peptides were recorded in 15–17 segments, with each succeeding segment representing a proportional reduction in reflectron voltage. About 300–500 laser shots were averaged per segment. All segments were pasted, calibrated, and smoothed to a final spectrum under computer control by the flexAnalysis 2.4 software.

CKX activity assay

For determination of electron acceptor preference of AtCKX proteins, the enzymatic activity was assayed with 250 µM iPR as a substrate without the acceptor (in the presence of oxygen) and with the use of following electron acceptors (500 µM): 2,6-dichlorophenol indophenol (DCPIP; LOBA Feinchemie, Fischamend, Austria), 2,3-dimetoxy-5-methyl-1,4-benzoquinone (Q_0; Sigma, St. Louis, MO, USA), ferricyanide (Lachema, Brno, Czech Republic), 3',5'-dimethoxy-4-hydroxyacetophenone, cytochrome *c*, 2,3,5-triphenyl-tetrazolium chloride (all from Sigma), NAD^+ (Fluka, Buchs, Switzerland). For the assessment of substrate specificity 500 µM Q_0 as an electron acceptor was used with a variety of substrates (200 µM): N^6-(2-isopentenyl)adenine (iP), N^6-(2-isopentenyl)adenine 9-riboside (iPR), kinetin (K), kinetin riboside (KR) (all from Sigma), N^6-(2-isopentenyl)adenine 9-

glucoside (iP9G), N^6-(2-isopentenyl)adenine 9-riboside-5'-monophosphate (iPMP), *trans*-zeatin (tZ) (from OlChemIm, Olomouc, Czech Republic).

Cytokinin dehydrogenase activity was measured using a modified end-point method described earlier (Frébort *et al*., 2002). The reaction mixture (total volume of 0.6 ml in 1.5 ml tube) consisted of McIlvaine buffer (0.1 M citric acid and 0.2 M Na_2HPO_4), pH 5.0 to 7.5, electron acceptor, substrate (dissolved in DMSO, final concentration 2.5%), and an appropriate concentration of the enzyme sample. Incubation time at 37 °C was 1-16 h, depending on the enzyme activity. The enzymatic reaction was stopped by 0.3 ml of 40% trichloroacetic acid, followed by an addition of 0.2 ml of 4-aminophenol (2% solution in 6% trichloroacetic acid) and the samples were then centrifuged at 19500*g* for 5 min to remove protein precipitate. The absorption spectrum in the range of 300-500 nm was recorded using Agilent 8345 diode array spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) to determine the concentration of product Schiff base specific for a given substrate (ε_{352} = 15.2 mM^{-1} cm^{-1} for iP, iPR, iPMP and iP9G, ε_{352} = 3.4 mM^{-1} cm^{-1} for tZ, ε_{380} = 4.0 mM^{-1} cm^{-1} for K and KR). Each reaction was repeated twice.

Monitoring of substrate cleavage by capillary electrophoresis

An off-line capillary electrophoresis (CE) assay method was developed to determine the ability of AtCKX enzymes to degrade cytokinin nucleotides. The reaction mixture consisted of 50 mM MES/Tris buffer (pH 5.0), 500 μM Q_0 as an electron acceptor, a mixture of nine cytokinin nucleotides, mono-, di- and triphosphates of iP, tZ and cZ, 100 μM each and the enzyme sample. The reaction at 37 °C was followed for 3 h in 30 min intervals by injections of the reaction mixture aliquots without stopping the reaction. All the experiments were performed using an Agilent 3D CE System (Agilent Technologies, Waldbronn, Germany) equipped with a diode array UV–Vis detector. Experimental data were collected and analyzed with 3D CE ChemStation Software. Separations were carried out in bare-fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). Total length of the capillary was 64.5 cm (56 cm effective), 50 μm I.D. A new capillary was flushed with 1 M NaOH (15 min), deionized water (15 min) and equilibrated with background electrolyte (20 min). After each run the capillary was rinsed with 1 M NaOH (1 min), deionized water (1 min) and background electrolyte (2 min). Basic running buffer was used and the analytes were separated as anions in positive mode. Separations were carried out at 30 kV, the capillary cassette was thermostated to 20 °C. All quantifications were done at 268 nm using corrected peak areas and uridine monophosphate as an internal standard.

Acknowledgements

This study was supported by research grants from the Czech Science Foundation (GACR) 522/08/H003 and the Ministry of Education, Youth and Sports MSM6198959216.

References

Bae, E., Bingman, C.A., Bitto, E., Aceti, D.J., Philips Jr., G.N., 2008. Crystal structure of *Arabidopsis thaliana* cytokinin dehydrogenase. Proteins 70, 303-306.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783-795.

Bilyeu, K.D., Cole, J.L., Laskey, J.G., Riekhof, W.R., Esparza, T.J., Kramer, M.D., Morris, R.O., 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol. 125, 378-386.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brownlee, B.G., Hall, R.H., Whitty, C.D., 1975. 3-Methyl-2-butenal: an enzymatic degradation product of the cytokinin N^6-(Δ^2-isopentenyl)adenine. Can. J. Biochem. 53, 37-41.

Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005-1016.

Frébort, I., Šebela, M., Galuszka, P., Werner, T., Schmülling, T., Peč, P., 2002. Cytokinin oxidase/dehydrogenase assay: optimized procedures and applications. Anal. Biochem. 306, 1-7.

Frébortová, J., Fraaije, M.W., Galuszka, P., Šebela, M., Peč, P., Hrbáč, J., Bilyeu, K.D., English, J.T., Frébort, I., 2004. Catalytic reaction of cytokinin dehydrogenase: Preference for quinones as electron acceptors. Biochem. J. 380, 121-130.

Frébortová, J., Galuszka, P., Werner, T., Schmülling, T., Frébort, I., 2007. Functional expression and purification of cytokinin dehydrogenase from *Arabidopsis thaliana* (AtCKX2) in *Saccharomyces cerevisiae*. Biol. Plant. 51, 673-682.

Frébortová, J., Novák, O., Frébort, I., Jorda, R., 2010. Degradation of cytokinins by maize cytokinin dehydrogenase is mediated by free radicals generated by enzymatic oxidation of natural benzoxazinones. Plant J. 61, 467-481.

Galuszka, P., Frébort, I. Šebela, M., Peč, P., 2000. Degradation of cytokinins by cytokinin oxidases in plants. Plant Growth Regul. 32, 315-327.

Galuszka, P., Frébort, I., Šebela, M., Sauer, P., Jacobsen, S., Peč, P., 2001. Cytokinin oxidase or dehydrogenase? Mechanism of the cytokinin degradation in plants. Eur. J. Biochem. 268, 450-461.

Galuszka, P., Popelková, H., Werner, T., Frébortová, J., Pospíšilová, H., Mik, V., Köllmer, I., Schmülling, T., Frébort, I., 2007. Biochemical characterization and histochemical localization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabaccum* L. J. Plant Growth Regul. 26, 255-267.

Hare, P.D., van Staden, J., 1994. Cytokinin oxidase: Biochemical features and physiological significance. Phys. Plant. 91, 128-136.

Kopečný, D., Pethe, C., Šebela, M., Houba-Herin, N., Madzak, C., Majira, A., Laloue, M., 2005. High-level expression and characterization of *Zea mays* cytokinin oxidase/dehydrogenase in *Yarrowia lipolytica*. Biochimie 87, 1011-1022.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Laskey, J.G., Patterson, P., Bilyeu, K., Morris, R.O., 2003. Rate enhacement of cytokinin oxidase/dehydrogenase using 2,6-dichloroindophenol as an electron acceptor. Plant Growth Regul. 40, 189-196.

Lin-Cereghino, J., Lin-Cereghino, G.P., 2007. Vectors and strains for expression, in: Cregg, J.M. (Ed.), *Pichia* Protocols. Humana Press, Totowa, New Jersey, pp. 11-27

Mok, D.W., Mok, M.C., 2001. Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 89-118.

Morris, R.O., Bilyeu, K.D., Laskey, J.G., Cheikh, N.N., 1999. Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. Biochem. Biophys. Res. Commun. 255, 328-333.

Nakai, K., Horton, P., 1999. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. 24, 34-36.

Nakamura, K., Matsuoka, K., 1993. Protein targeting to the vacuole in plant cells. Plant Physiol. 101, 1-5.

Pačes, V., Werstiuk, E., Hall, R.H., 1971. Conversion of N^6-(Δ^2-isopentenyl)adenosine to adenosine by enzyme activity in tobacco tissue. Plant Physiol. 48, 775-778.

Popelková, H., Galuszka, P., Frébortová, J., Bilyeu, K.D., Frébort, I., 2004. Cytokinin dehydrogenase: Characterization and structure homology modelling of the flavoprotein catabolizing plant hormones cytokinins, in: Pandalai S.G. (Ed.), Recent Research Developments in Proteins, vol. 2. Transworld Research Network, Kerala, India, pp. 63–81.

Schägger, H., von Jagov, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368-379.

Schmülling, T., Werner, T., Riefler, M., Krupková, E., Bartrina y Manns, I., 2003. Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. J. Plant Res. 116, 241-252.

Shevchenko, A., Tomas, H., Havliš, J., Olsen, J.V., Mann, M., 2007. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 2856–2860.

Šebela, M., Štosová, T., Havliš, J., Wielsch, N., Thomas, H., Zdráhal, Z., Shevchenko, A., 2006. Thermostable trypsin conjugates for high throughput proteomics: synthesis and performance evaluation. Proteomics 6, 2959-2963.

Šmehilová, M., Galuszka, P., Bileyu, 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. 6, 2701-2712.

Takahama, U., 2004. Oxidation of vacuolar and apoplastic phenolic substrates by peroxidase: Physiological significance of the oxidation reactions. Phytochem. Rev. 3, 207-219.

Vitale, A., Raikhel, N.V., 1999. What do proteins need to reach different vacuoles? Trends Plant Sci. 4, 149-155.

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.

Werner, T., Motyka, V., Strnad, M., Schmülling, T., 2001. Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. U.S.A. 98, 10487-10492.

Werner, T., Motyka, V., Laucou, V., Smets, R., van Onckelen, H., 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15, 1-20.

Werner, T., Köllmer, I., Bartrina y Manns, I., Holst, K., Schmülling, T., 2006. New insights into the biology of cytokinin degradation. Plant. Biol. 8, 371-381.

Part 3

High-level expression of recombinant cytokinin dehydrogenases from *Arabidopsis thaliana* in a constitutive *Pichia pastoris* expression system

Abstract

Cytokinins homeostasis in tissues of many lower and higher plants is in large part controlled by the activity of cytokinin dehydrogenase (CKX, EC 1.5.99.12) that catalyzes the irreversible cleavage of N^6-side chain of cytokinins. The *CKX* gene family of *Arabidopsis thaliana* comprise seven members, four of which code for apoplastic proteins, two for vacuolar and one cytosolic. This study presents the production of an apoplastic enzyme CKX2 from *Arabidopsis thaliana* in *Pichia pastoris* constitutive expression system. Recombinant protein was fused with His-tag domain on its *C*-terminus, *N*-terminus and on both termini in order to facilitate purification and the influence of such fusions on enzyme's activity was determined. Large-scale expression in 15 litres fermentor was established with glycerol and glucose as the carbon source.

Introduction

Cytokinin dehydrogenase (CKX; EC 1.5.99.12) is the key enzyme in catabolism of plant hormone cytokinin regulating in large part its homeostasis (Hare and van Staden, 1994; Galuszka *et al.,* 2000). It catalyzes an irreversible cleavage of the N^6-side chain of cytokinins to form adenine and a side chain derived aldehyde (Brownlee *et al.,* 1975). The CKX enzyme is a flavoprotein, with covalently bound FAD molecule (Popelková *et al.* 2006), that prefers electron acceptors other than molecular oxygen as the primary electron acceptor (Galuszka *et al.,* 2001; Bileyu *et al.,* 2001; Laskey *et al.,* 2003; Frébortová *et al.,* 2010).

In different higher plant species, CKX proteins are encoded by small gene families with a varying number of members (Werner *et al.,* 2006). In the genome of *Arabidopsis thaliana* seven distinct CKX-encoding genes (*AtCKX1-AtCKX7*) were identified. Individual AtCKX proteins share conserved FAD-binding and substrate-binding domains but their amino acid identity is between 34.3 and 65.9% therefore they differ in their biochemical properties, in regulation of their expression and in subcellular localization (Schmülling *et al.,* 2003; Popelková *et al.,* 2004). *N*-terminal sequences of AtCKX proteins indicate apoplastic targeting of four of them (AtCKX2, AtCKX4, AtCKX5 and AtCKX6), two proteins (AtCKX1 and AtCKX3) are directed to vacuoles and AtCKX7 that lacks any signal peptide is presumably localized to cytosol.

The most studied and most abundant CKX enzyme in *Arabidopsis* is an apoplastic protein AtCKX2. It was heterologously expressed in *Sacharomyces cerevisiae* (Werner *et al.,* 2001; Frébortová *et al.,* 2007) and *Pichia pastoris* (Bilyeu *et al.,* 2001). Only in the former case the recombinant protein was secreted into the culture medium, purified to homogeneity and characterized (Frébortová *et al.,* 2007).

Pichia pastoris was recognized nearly 40 years ago as a potential source for production of single-cell proteins and over the years it has developed into a highly successful system for the production of a variety of heterologous proteins (Lin-Cereghino and Cregg, 2000). There are several features of *Pichia* that decide of its popularity as an expression host. The key advantages include: easy manipulation at the molecular genetic level (e.g. gene targeting, high-frequency DNA transformation, cloning by functional complementation), the ability to express foreign proteins at high levels, either intracellularly or extracellularly, the capability of performing many eukaryotic post-translational modifications (such as glycosylation, disulfide-bond formation and proteolytic processing), growth to high cell densities on simple, inexpensive media in a very wide pH range (from 3 to 7), a lack of endotoxins and the low level of native secreted proteins that simplifies the purification of many secreted recombinant proteins (Cos *et al.,* 2006; d'Anjou and Daugulis, 1997). And since *Pichia* does not ferment sugars or other carbon sources and prefers respiratory growth turning the carbon source into a biomass it can be cultured at extremely high densities in the controlled environment of the

fermentor. Most expression vectors for *Pichia* rely on its ability to grow on methanol and the existence of a strong and tightly regulated promoter from the alcohol oxidase 1 gene, *AOX1* (Cregg *et al*., 2007). However to avoid the need of methanol induction alternative promoters can be used, like the glucose-inducible strong constitutive glyceraldehyde 3-phosphate dehydrogenase (*GAP*) gene promoter (Waterham *et al*., 1997).

In our study we focused on the constitutive extracellular expression of AtCKX2 in *Pichia pastoris* under the GAP promoter with respect to a high-level production in fermentor cultures. To achieve protein secretion from the yeast cell, *S. cerevisiae* α-factor prepro peptide was used. Also the recombinant protein was fused with His-tag domain on its *C*-terminus, *N*-terminus and on both termini in order to facilitate purification. However histidine fusions influenced enzyme's activity and the extent of such changes was determined. The main objective of the AtCKX2 production was to utilize the enzyme in biotechnological applications like cytokinin biosensor.

Results and discussion

Comparison of differently tagged proteins

AtCKX2 enzyme was first expressed in pGAPZαA vector without any tags to confirm the protein secretion to *Pichia* growth medium by CKX activity assay. Native secretion signal of the protein was replaced by the 85 amino acid α-factor prepro peptide from *S. cerevisiae*, as it has proven to be a potent and easily removed secretion signal (Lin-Cereghino and Cregg, 2000; Lin-Cereghino *et al*., 2002). As a result efficient release of the expressed AtCKX2 protein into the growth medium was achieved. In order to facilitate purification, recombinant protein was further fused with *C*-terminal His-tag domain, present in the pGAPZαA vector. However, such fusion was shown to decrease CKX activity, therefore a novel vector was prepared from pGAPZαA by inserting a coding sequence for 10x His from pET16b between the α-factor coding sequence and the multiple cloning site. *AtCKX2* gene was subsequently cloned into pGAPZαA$(His)_{10}$ with and without *C*-terminal His-tag fusion and expressed in *Pichia pastoris* X-33. All four proteins were simultaneously cultivated for 60 h in 250 ml shake flasks in YPD medium. Differently tagged proteins were then compared in their specific activities towards iP and iPR with DCPIP in pH 6.5 and Q_0 in pH 5.0, respectively (Table 1). It is noticeable that *N*-terminal His-tag slightly increases enzymatic activity of AtCKX2 whereas *C*-terminally tagged protein is the least active (activity lowered more than twice). Fusion domains on both ends of the recombinant protein also reduce CKX activity compare to the tag less enzyme but not as much as only *C*-terminal domain.

Table 1. Comparison of specific activities of AtCKX2 enzyme bearing different fusion domains.

	Specific activity (nkat/mg)	
	Q_0, iPR, pH 5.0	DCPIP, iP, pH 6.5
AtCKX2	2.66	15.68
$(His)_{10}$-AtCKX2	3.02	16.77
AtCKX2-$(His)_6$	1.43	5.87
$(His)_{10}$-AtCKX2-$(His)_6$	2.10	7.55

Fed-batch production of recombinant AtCKX2

Expression of heterologous proteins in fermentor cultures results in a relatively high concentration of vacuolar proteases such as proteinase A (*pep4*) due to the combination of high-cell density and lysis of a small percentage of cells. To reduce such degradation of recombinant proteins, *Pichia* strains deficient in vacuolar proteases should be used (Lin-Cereghino and Lin-Cereghino, 2007). Therefore an auxotrophic and protease-deficient strain SMD1168 (*his4*, *pep4*) was chosen and new vector construct was prepared. The expression cassette of pGAPZαA$(His)_{10}$::AtCKX2 containing constitutive GAP promoter and *AtCKX2* gene was inserted into *HIS4*-based vector pPIC9K. After *Pichia* transformation the most active clone was selected on the basis of Geneticin® resistance, conferred by the *Kan* gene in the expression vector. Multiple integrated copies of pPIC9K can increase the Geneticin® resistance level from 0.5 mg/ml up to 4 mg/ml. By this approach, strains carrying up to 30 copies of an expression cassette have been isolated (Clare *et al*. 1991). To determine the copy number of *CKX2* genes integrated into the yeast genome a qPCR was used with *aox1* gene as an endogenous control and *Pichia* transformant bearing pGAPZαA::AtCKX2 plasmid as reference. Purified genomic DNA was digested with *NcoI* enzyme and subjected to qPCR analysis. *Pichia* transformed with the modified pPIC9K vector under the control of GAP promoter, grown on 1.75 mg/ml of Geneticin® and demonstrating the highest activity of all screened clones, was shown to have 4 copies of the *AtCKX2* gene. The expression level of AtCKX2 enzyme from this vector is 2.5 times higher compared to the pGAPZαA$(His)_{10}$::AtCKX2 therefore it was selected for large scale expression in a fermentor.

The AtCKX2 expression in the fermentor was carried in fed-batch mode (Figure 1) with 50% glucose containing biotin, defoamer and trace salts as a feeding medium. Cell yield was from 70 up to 180 g/l dry cell weight. The CKX activity began to increase shortly after feed-batch start and continued to grow till the end of the fermentation process.

The constitutive expression system allows safe handling of the *P. pastoris* production system, avoiding the hazardous use of methanol, which is especially appreciated in large scale protein production. However further optimization of the cultivation conditions are needed for the described system, possibly concerning continuous fermentation that was proved to be even more effective, due to the reduction of yeast proteases concentration (Goodrick *et al.,* 2001).

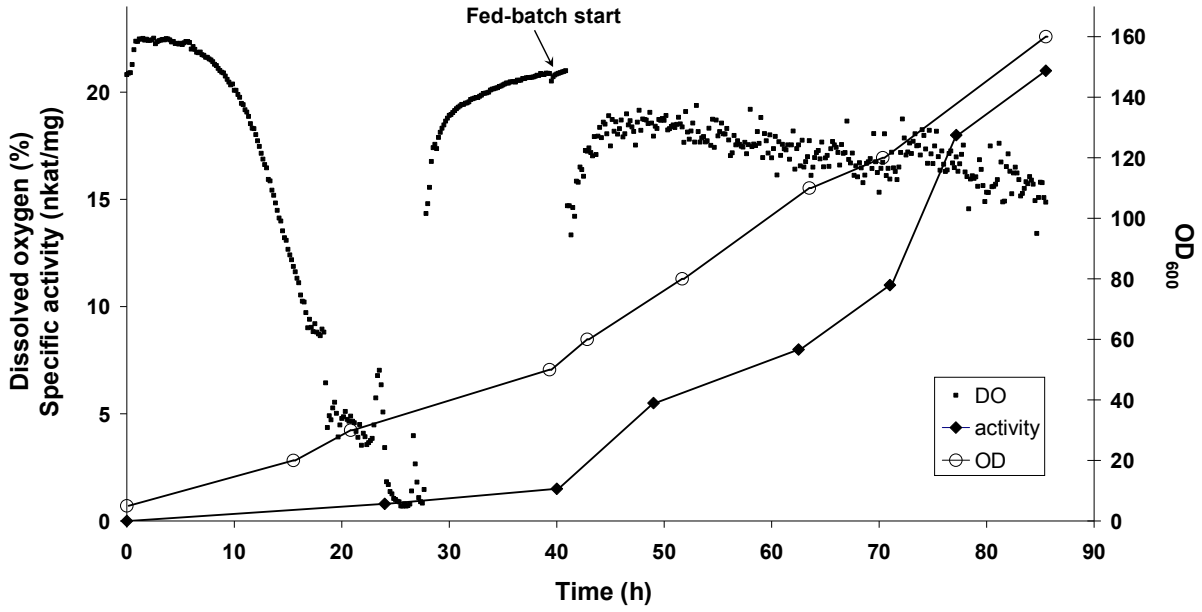


Figure 1. Constitutive AtCKX2 expression in fed-batch culture of *Pichia pastoris* (SMD1168, *His*$^-$, modified pPIC9K vector with GAP promoter). A 50% glucose solution was fed at the rate of 0.2 ml/min. DO – dissolved oxygen, OD – optical density at 600 nm.

Experimental

Construction of expression vectors

The leaves of transgenic tobacco overexpressing *AtCKX2* gene (Werner *et al.,* 2001) were the starting material for RNA isolation with Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was carried out with the RevertAid™ H Minus M-MuLV Reverse Transcriptase protocol (Fermentas, Vilnius, Lithuania). Specific primers (Table 2) were designed so that resulting amplicons would be missing *N*-terminal fragment of 66 nucleotides predicted to be a signal sequence (SignalP 3.0 Server, Bendtsen *et al,* 2004) and possibly could be *C*-terminally fused with His-tag domain of the expression vector. The *AtCKX2* gene was amplified with the use of Phusion DNA Polymerase (Finnzymes, Espoo, Finland) in a TGradient Thermocycler (Biometra, Goettingen, Germany). The amplification program was 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 60 s at 55 °C, 30 s at 72 °C; and terminated by 10 min at 72 °C. The gene was further cloned into pGAPZαA shuttle vector (Invitrogen) or pGAPZαA$(His)_{10}$ vector, carrying an additional *N*-terminal His-tag sequence precloned from pET16b vector (Novagen, Madison, WI, USA). Plasmid constructs were transformed into *E. coli* TOP10F (Invitrogen) by electroporation at 1.8 kV and transformants were selected on the basis of zeocin resistance.

Table 2. Sequences of the primers used for cloning of AtCKX2 gene into *Pichia* transformation vectors.

Vector	Primer sequence in 5´ to 3´-end direction	Restriction site
pGAPZαA	ggaattcATTAAAATTGATTTACCTAAAT	*EcoRI*
	gctctagatCAAAAGATGTCTTGCCC	*XbaI*
pGAPZαA$(His)_{10}$	ggaattccatatgATTAAAATTGATTTACCTAAAT	*NdeI*
	gctctagatCAAAAGATGTCTTGCCC	*XbaI*
pGAPZαA-$(His)_6$	ggaattcATTAAAATTGATTTACCTAAAT	*EcoRI*
	gctctagaCAAAAGATGTCATTGCC	*XbaI*
pGAPZαA$(His)_{10}$-$(His)_6$	ggaattccatatgATTAAAATTGATTTACCTAAAT	*NdeI*
	gctctagaCAAAAGATGTCATTGCC	*XbaI*

Transformation of Pichia pastoris

The plasmid constructs pGAPZαA(His)$_{10}$::AtCKX2 were used for integration into the *Pichia pastoris* X-33 (Invitrogen) genome after linearization with *AvrII* (NEB, Ipswich, MA, USA) that cuts once within GAP promoter region. Yeasts were transformed by electroporation at 1.5 kV according to the manufacturer's protocol and spread onto YPDS plates (1% yeast extract, 2% peptone, 2% D-glucose, 1 M sorbitol, 2% agar) containing 100 mg/l zeocin (Duchefa Biochemie B.V., Haarlem, The Netherlands). Selected transformants were picked up and grown for one day in 2 ml of YPD medium (1% yeast extract, 2% peptone, 2% D-glucose) with 100 mg/l zeocin, at 30 ºC with extensive shaking at 230 rpm. Subsequently, the pGAPZαA(His)$_{10}$::AtCKX2 transformants were transferred into 50 ml of YPD medium without zeocin buffered to pH 7.2 with 0.1 M potassium phosphate buffer. After 48 h cultivation at 28 ºC with 230 rpm shaking, yeast cells were removed by centrifugation at 5000*g* for 10 min and the CKX activity was measured in the cell-free medium.

Preparation of pPIC9K vector under control of constitutive GAP promoter

The plasmid construct pGAPZαA(His)$_{10}$::AtCKX2 and pPIC9K vector (Invitrogen) were subjected to partial digestion with *BglII* (Takara) and *BshTI* (Fermentas). Digestion products of the expected size (approx. 8 kb for pPIC9K and 2.4 kb for pGAPZαA(His)$_{10}$::AtCKX2) were further ligated together and transformed into *E. coli* TOP10F (Invitrogen) by electroporation at 1.8 kV. Selected plasmid constructs pPIC9K::AtCKX2 were linearized with *AvrII* (NEB) before integration into *P. pastoris* SMD1168 (Invitrogen) genome. His$^+$ transformants were grown on MD plates (1.34% yeast nitrogen base without amino acids (Difco™, Detroit, MI, USA), $4 \cdot 10^{-5}$% biotin, 2% D-glucose, 2% agar). Screening for multicopy inserts was carried on YPD plates (1% yeast extract, 2% peptone, 2% D-glucose, 1.5% agar) containing various concentrations (from 0.5 to 3 mg/ml) of Geneticin® (G-418 sulfate) (Calbiochem, Merck, Darmstadt, Germany). Selected transformants were picked up and grown as described above but with a proper concentration of Geneticin instead of zeocin and further the CKX activity in the cell-free medium was measured as follows.

CKX activity assay

A modified end-point method (Frébort *et al.* 2002) was used for cytokinin dehydrogenase activity measurements. The reaction mixture (total volume of 0.6 ml in 1.5 ml tube) consisted of 100 mM McIlvaine buffer, pH 5.0 or 6.5, electron acceptor, substrate (dissolved in DMSO, final concentration 2.5%), and an appropriate concentration of the enzyme sample. Incubation time at 37 °C was 0.5-1 h, depending on the enzyme activity. As electron acceptors 500 μM 2,3-dimetoxy-5-methyl-1,4-benzoquinone (Q_0; Sigma, St. Louis, MO, USA) and 2,6-dichlorophenol indophenol (DCPIP;

LOBA Feinchemie, Fischamend, Austria) were used in pH 5.0 and 6.5, respectively. Cytokinins degraded by AtCKX2 in the reaction mixture were 250 μM N^6-(2-isopentenyl)adenine (iP) and N^6-(2-isopentenyl)adenine 9-riboside (iPR) in pH 6.5 and 5.0, respectively. To stop the enzymatic reaction 0.3 ml of 40% trichloroacetic acidwas added, followed by 0.2 ml of 4-aminophenol (2% solution in 6% trichloroacetic acid). Protein precipitate was removed from the samples by centrifugation at 19500*g* for 5 min. The absorption spectrum in the range of 300-500 nm was recorded using Agilent 8345 diode array spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) to determine the concentration of product Schiff base specific for a given substrate (ε_{352} = 15.2 mM^{-1} cm^{-1} for iP, iPR).

Protein content in enzyme samples was measured according to Bradford (1976) with bovine serum albumin as a standard.

Estimation of AtCKX2 gene copy number

To establish how many copies of *AtCKX2* gene was integrated into pPIC9K vector a real-time PCR experiment was designed. Yeast genomic DNA isolated with the use of MasterPure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) and digested with *NcoI* (Fermentas) served as a template. Primers for *ckx2* and *aox1* genes were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). The real-time reaction mixtures contained diluted DNA samples, POWER SYBR Green PCR Master Mix and 300 nM of each primer. All DNA samples were run in four technical replicates on the StepOne-Plus Real-Time PCR System using a default program (Applied Biosystems). Cycle threshold values were normalized with respect to the alcohol oxidase 1 gene.

High-cell density fermentation

Fermentation experiments were performed in a 15 litre R'ALF Plus Duet fermentor (Bioengineering AG, Wald, Switzerland) with a 10 l working volume and control modules for pH, temperature and dissolved oxygen. The inoculum was grown in shake flask at 30 °C with orbital shaking at 230 rpm, in 200 ml of medium containing 13.4 g/l of yeast nitrogen base without amino acids (Difco™), 0.1 M potassium phosphate buffer (pH 7.2) and 2% D-glucose. After 24 to 40 h cultivation, until the cell density reached an OD_{600} of >10, the cells from the flask were used to inoculate the medium in the fermentor that had the same composition as inoculum but with 1% glycerol as a carbon source and pH 6.5. The process temperature was maintained at 30 °C and pH was controlled by the addition of 5 M KOH. The pH was measured with a Mettler Toledo pH electrode 405-DPAS-SC-K8S/325 (Urdorf, Switzerland). The impeller speed was set to 800 rpm and the air flow was 300 l/h. The oxygen concentration was monitored with a Mettler Toledo InPro® 6950/6900

O_2 Sensor. To reduce foam formation, a KFO 673 defoamer (Emerald Performance Materials, Cheyenne, WY, USA) was added. Fed-batch fermentation was initiated after about 40 h, when a dissolved oxygen spike appeared indicating the depletion of the initial glycerol and the need for feeding. The fed-batch medium consisted of (per litre of deionized water): 500 g D-glucose, 2.4 mg D-biotin, 0.2% defoamer and 4 ml trace salts solution (per litre of deionized water: H_3BO_3 0.02 g, $CuSO_4 \cdot 5H_2O$ 2 g, KI 0.1 g, $MnSO_4 \cdot H_2O$ 3 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, $ZnSO_4 \cdot 7H_2O$ 17.8 g, $CoCl_2$ 0.92 g) and it was fed at a rate of 0.2 ml/min. In order to monitor culture density and CKX activity over time samples were taken in different time points. The fermentation process was stopped after about 50 h of feeding and yeast cells were removed by centrifugation at 4600*g* for 40 min at 4 °C. The cell-free medium was concentrated to about 60 ml by ultrafiltration in a VivaFlow 50 system (Sartorius Stadius Biotech GmbH, Goettingen, Germany) with the 30 kDa membrane cut-off.

References

d'Anjou, M.C., Daugulis A.J., 1997. A model based feeding strategy for fed-batch fermentation of recombinant *Pichia pastoris*. Biotechnol. Techniques 11**,** 865-868.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783-795.

Bilyeu, K.D., Cole, J.L., Laskey, J.G., Riekhof, W.R., Esparza, T.J., Kramer, M.D., Morris, R.O., 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol. 125, 378-386.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brownlee, B.G., Hall, R.H., Whitty, C.D., 1975. 3-Methyl-2-butenal: an enzymatic degradation product of the cytokinin N^6-(Δ^2-isopentenyl)adenine. Can. J. Biochem. 53, 37-41

Clare, J.J., Romanos, M.A., Rayment, F.B., 1991. Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* containing multiple gene copies. Gene 105, 205-212.

Cregg, J.M.(ed.), 2007. *Pichia* Protocols. Humana Press, Totowa, New Jersey.

Cos, O., Ramón, R., Montesinos, J.L., Valero, F., 2006. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. Microb. Cell Fact. 5:17.

Frébort, I., Šebela, M., Galuszka, P., Werner, T., Schmülling, T., Peč, P., 2002. Cytokinin oxidase/dehydrogenase assay: optimized procedures and applications. Anal. Biochem. 306, 1-7.

Frébortová, J., Galuszka, P., Werner, T., Schmülling, T., Frébort, I., 2007. Functional expression and purification of cytokinin dehydrogenase from *Arabidopsis thaliana* (AtCKX2) in *Saccharomyces cerevisiae*. Biol. Plant. 51, 673-682.

Frébortová, J., Novák, O., Frébort, I., Jorda, R., 2010. Degradation of cytokinins by maize cytokinin dehydrogenase is mediated by free radicals generated by enzymatic oxidation of natural benzoxazinones. Plant J. 61, 467-481.

Galuszka, P., Frébort, I. Šebela, M., Peč, P., 2000. Degradation of cytokinins by cytokinin oxidases in plants. Plant Growth Regul. 32, 315-327.

Galuszka, P., Frébort, I., Šebela, M., Sauer, P., Jacobsen, S., Peč, P., 2001. Cytokinin oxidase or dehydrogenase? Mechanism of the cytokinin degradation in plants. Eur. J. Biochem. 268, 450-461.

Goodrick, J.C., Xu, M., Finnegan, R., Schilling, S., Schiavi, S., Hoppe, H., Wan, N.C., 2001. High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system. Biotechnol. Bioeng. 74, 492-497.

Hare, P.D., van Staden, J., 1994. Cytokinin oxidase: Biochemical features and physiological

significance. Phys. Plant. 91, 128-136.

Laskey, J.G., Patterson, P., Bilyeu, K.D., Morris, R.O., 2003. Rate enhacement of cytokinin oxidase/dehydrogenase using 2,6-dichloroindophenol as an electron acceptor. Plant Growth Regul. 40, 189-196.

Lin-Cereghino, J., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol.* Rev. 24, 45-66.

Lin-Cereghino, G.P., Lin-Cereghino, J., Ilgen, Ch., Cregg, J.M., 2002. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Curr. Opinion Biotechnol. 13, 329-332.

Lin-Cereghino, J., Lin-Cereghino, G.P., 2007. Vectors and strains for expression, in: Cregg, J.M., Pichia Protocols. Humana Press, Totowa, New Jersey, pp. 11-27

Popelková, H., Galuszka, P., Frébortová, J., Bilyeu, K.D., Frébort, I., 2004. Cytokinin dehydrogenase: Characterization and structure homology modelling of the flavoprotein catabolizing plant hormones cytokinins, in: Pandalai S.G. (ed), Recent Research Developments in Proteins, vol. 2, Transworld Research Network, Kerala, India, pp. 63–81.

Popelková, H., Fraaije, M.W., Novák, O., Frébortová, J., Bilyeu, K.D., Frébort, I., 2006. Kinetic and chemical analyses of the cytokinin dehydrogenase-catalysed reaction: correlations with the crystal structure. Biochem. J. 398, 113-124 .

Schmülling, T., Werner, T., Fiedler, M., Krupková, E., Bartrina, I., 2003 Structure and fiction of cytokinin oxidase/dehydroganase genes of maize, rice, *Arabidopsis* and other species. J. Plant Res. 116, 241-252.

Waterham, H.R., Digan, M.E., Koutz, P.J., Lair, S.V., Cregg, J.M., 1997. Isolation of the *Pichia pastoris* glyceraldehyde 3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 186, 37-44.

Werner, T., Motyka, V., Strnad, M., Schmülling, T., 2001 Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. U.S.A. 98, 10487-10492.

Werner, T., Köllmer, I., Bartrina, I., Holst, K., Schmülling, T., 2006. New insights into the biology of cytokinin degradation. Plant. Biol. 8, 371-381.

Curriculum vitae

Personal data

First name:	Marta
Last name:	Kowalska
Date of birth:	July 31, 1980
Place of birth:	Zambrów
Nationality:	Polish
E-mail:	marta.kowalska@email.cz

Education and study

2006 – (2010) Ph.D. Study program in Biochemistry at the Faculty of Science, Palacký University in Olomouc; theme "Cytokinins metabolism and action" (advisor Ivo Frébort).

2004 – 2006 Ph.D. Study program in Physical Chemistry at the Chemical Faculty, Gdańsk University of Technology, Gdańsk; interrupted.

1999 – 2004 Graduated in Biotechnology (M.Sc, M.Eng.) in 2004 at the Chemical Faculty, Gdańsk University of Technology, Gdańsk; Diploma thesis "Use of variable numbers of tandem repeat VNTR in differentiation of *Staphylococcus aureus* strains".

International scholarship

May – June 2010 University of Warwick, Coventry, UK – Palacký University student internship with participation on "Fabrication of cytokinins biosensor" project.

April – July 2009 University of Warwick, Coventry, UK – Research scholar stay with participation on "Fabrication of cytokinins biosensor" project.

List of author's publications

Chrzanowski, W., **Kowalska, M.**, Trznadel, M., Grzybkowski, W., 2005, Studies on water – polyethylene glycol – sulfates(VI) ternary systems used in conservation of wooden historical artefacts, *Annals of the Polish Chemical Soc.,* 1, 376-380.

Ryparová, O., Petr, J., **Kowalska, M.**, Znaleziona, J., M., Ranc, V., Knob, R., Maier, V., Frébort, I., Ševčík, J., 2008, Analýza mikroorganismů metodou kapilární elektroforézy. *Chemické listy*, 102, 1121−1126.

Petr, J., Ryparová, O., Ranc, V., Hinnerová, P., Znaleziona, J., **Kowalska, M.**, Knob, R., Maier, V., Frébort, I., Lemr, K., Ševčík, J., 2009, Assessment of CE for the identification of microorganisms. *Electrophoresis* 30*,* 444-449.

Šmehilová, M., Galuszka, P., Bileyu, 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, 2701-2712.

Kowalska, M., Galuszka, P., Frébortová, J., Šebela M., Béres, T., Hluska, T., Šmehilová, M., Bilyeu, K.D., Frébort, I., 2010, Non-secreted cytokinin dehydrogenases of *Arabidopsis thaliana*: Heterologous expression, purification and properties. *Phytochemistry*, in press

Kowalska, M., Tian, F., Dale, N., Napier, R., Šmehilová, M., Galuszka, P., Frébort, I., 2010, Towards cytokinin biosensor. (Manuscript)

Frébort, I., **Kowalska, M.**, Hluska, T., Galuszka, P., Frébortová, J., 2010, Darwin Review. Evolution of cytokinin biosynthesis and degradation. *J. Exp. Bot*., (invited review, manuscript)

Conferences

2010

Kowalska, M., Galuszka, P., Frébortová, J., Béres, T., Šmehilová, M., Frébort I.: Non-secreted cytokinin dehydrogenases of *Arabidopsis thaliana*. 20th International Conference on Plant Growth Substances, June 28 – July 2, 2010, Tarragona, Spain; poster PS15-10.

2009

Kowalska, M., Šmehilová, M., Galuszka, P., Frébort I.: Production of vacuolar cytokinin dehydrogenases from *Arabidopsis thaliana* in *Pichia pastoris.* Annual Main Meeting of the Society for Experimental Biology, June 28 - July 1, 2009, Glasgow, United Kingdom; poster P5.32; abstract in Comp. Biochem. Physiol. A: Mol. Integr. Physiol., 153 S1, S218, 2009.

Kowalska, M., Tian, F., Dále, N., Napier, R., Joseph, M., Frébort, I.: Developing cytokining biosensor. Auxins and Cytokinins in Plant Development – 3rd International Symposium, July 10 – 14, 2009, Prague, Czech Republic; poster P7-5.

Šmehilová, M., Galuszka, P., Bilyeu, K. D., Jaworek, P., **Kowalska, M.**, Šebela M., Sedlářová, M., English, J.T., Frébort I.: Characterisation of non-secreted cytokinin dehydrogenase from maize, comparison with apoplastic isoform. Auxins and Cytokinins in Plant Development – 3rd International Symposium, July 10 – 14, 2009, Prague, Czech Republic; poster P1-13.

2008

Šmehilová, M., Galuszka, P., **Kowalska, M.**, Bilyeu, K. D., Frébort I.: Biochemical and structural comparison of two differentially targeted cytokinin dehydrogenase enzymes. XIIth Meeting of Biochemists and Molecular Biologists, February 6 – 7, 2008, Brno, Czech Republic; lecture by Mária Šmehilová; abstract in: Book of Abstracts (Sborník přednášek a posterů), Masaryk University, Brno, p. 52; ISBN 978-80-210-4526-2.

Kowalska, M., Galuszka, P., Frébort I.: Engeneering of plant vacuolar proteins for production in *Pichia pastoris*. BioTech 2008 & 4th Swiss-Czech Symposium "Biopharmaceuticals: why use yeasts?", May 22 - 23, 2008, Wädenswill, Switzerland; poster no. 30; abstract in Conference proceedings, p. 73; ISBN 978-3-905745-14-6.

Kowalska, M., Šmehilová, M., Galuszka, P., Frébort I.: Expression and comparison of *Arabidopsis thaliana* cytokinin oxidase/dehydrogenase isoenzymes. Seminář k vědeckovýzkumnému záměru MŠM 6198959216, May 22 – 24, 2008, Karlov p. Pradědem, Czech Republic; Book of Abstracts p. 9, Palacký University, Olomouc.

Šmehilová, M., Galuszka, P., **Kowalska, M.**, Frébort I., Bilyeu, K. D.: Biochemical and structural comparison of two differentially targeted cytokinin dehydrogenase enzymes. 33rd FEBS Congress and

11th IUBMB Conference, June 28- July 3, 2008, Athens, Greece; poster PP7A-82; abstract in FEBS J. 275 S1, 320, 2008.

Kowalska, M., Šmehilová, M., Galuszka, P., Frébort I.: Expression of *Arabidopsis thaliana* vacuolar cytokinin oxidase/dehydrogenases in *Pichia pastoris.* XXI Joint Congress of the Czech and Slovak Societies for Biochemistry and Molecular Biology, September 14 - 17, 2008, České Budějovice, Czech Republic; poster P140; abstract in: Book of Abstracts (Sborník přednášek a posterů), JPM Tisk, Prague, p. 162, ISBN 80-86313-21-2.

Šmehilová, M., **Kowalska, M.**, Galuszka, P., Jaworek, P., Bilyeu, K. D., Frébort I.: Biochemical and structural comparison of two differentially targeted cytokinin dehydrogenase enzymes. XXI Joint Congress of the Czech and Slovak Societies for Biochemistry and Molecular Biology, September 14 - 17, 2008, České Budějovice, Czech Republic; poster P48; abstract in: Book of Abstracts (Sborník přednášek a posterů), JPM Tisk, Prague, p. 84, ISBN 80-86313-21-2.

2007

Kowalska, M., Šmehilová, M., Galuszka, P., Frébort I.: *Arabidopsis thaliana* cytokinin oxidase/dehydrogenase isoenzymes – cloning, expression and comparison of substrate specificity. XIth Meeting of Biochemists and Molecular Biologists, January 31 - February 1, 2007, Brno, Czech Republic; lecture by Marta Kowalska; abstract in: Book of Abstracts (Sborník přednášek a posterů), Masaryk University, Brno, p. 29; ISBN 978-80-210-4234-6.

Šmehilová, M., Galuszka, P., **Kowalska, M.**, Bilyeu, K. D., Frébort I.: Compartmentation of the enzymatic degradation of cytokinins in plants. 11th Days of Plant Physiology, July 9 – 12, 2007, Olomouc, Czech Republic; lecture by Mária Šmehilová; abstract in Bull. Czech Soc. Exp. Biol. Plants 7, 52, 2007.

Galuszka, P., **Kowalska, M.**, Šmehilová, M., Werner, T.: Substrate specificity and biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana*. International Plant Growth Substances Association, 19th Annual Meeting, July 21 - 25, 2007, Puerto Vallarta, Mexico; poster PS1618, Abstracts p. 147.

Kowalska, M., Šmehilová, M., Galuszka, P., Frébort I.: Functional heterologous expression of *Arabidopsis thaliana* cytokinin oxidase/dehydrogenase family. 2nd International symposium "Plant Growth Substances: Intracellular Hormonal Signaling and Agriculture Application", October 8 - 12, 2007, Kiev, Ukraine; poster, Abstracts p. 50.