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Research article

# Maize cytokinin dehydrogenase isozymes are localized predominantly to the vacuoles

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ABSTRACT

The maize genome encompasses 13 genes encoding for cytokinin dehydrogenase isozymes (CKXs). These enzymes are responsible for irreversible degradation of cytokinin plant hormones and thus, contribute regulating their levels. Here, we focus on the unique aspect of CKXs: their diverse subcellular distribution, important in regulating cytokinin homeostasis. Maize CKXs were tagged with green fluorescent protein (GFP) and transiently expressed in maize protoplasts. Most of the isoforms, namely ZmCKX1, ZmCKX2, ZmCKX4a, ZmCKX5, ZmCKX6, ZmCKX8, ZmCKX9, and ZmCKX12, were associated with endoplasmic reticulum (ER) several hours after transformation. GFP-fused CKXs were observed to accumulate in putative prevacuolar compartments. To gain more information about the spatiotemporal localization of the above isoforms, we prepared stable expression lines of all ZmCKX–GFP fusions in *Arabidopsis thaliana* Ler suspension culture. All the ER-associated isoforms except ZmCKX1 and ZmCKX9 were found to be targeted primarily to vacuoles, suggesting that ER-localization is a transition point in the intracellular secretory pathway and vacuoles serve as these isoforms' final destination. ZmCKX9 showed an ER-like localization pattern similar to those observed in the transient maize assay. Apoplastic localization of ZmCKX1 was further confirmed and ZmCKX10 showed cytosolic/nuclear localization due to the absence of the signal peptide sequence as previously reported. Additionally, we prepared GFP-fused N-terminal signal deletion mutants of ZmCKX2 and ZmCKX9 and clearly demonstrated that the localization pattern of these mutant forms was cytosolic/nuclear. This study provides the first complex model for spatiotemporal localization of the key enzymes of the cytokinin degradation/catabolism in monocotyledonous plants.



## 1. Introduction

The cytokinin plant hormones (CKs) play key roles in numerous developmental and physiological processes. The levels of the active hormone in plant organs, tissues, and cells must be precisely regulated to ensure correct biological function. Thus, CK *de novo* biosynthesis and activation, intracellular transport, and CK degradation must be tightly controlled within the cell. CKs' irreversible degradation is catalyzed by a group of enzymes known as cytokinin dehydrogenases (CKXs; EC 1.5.99.12).

From the chemical viewpoint, CKs are derivatives of adenine, carrying either isoprenoid or aromatic substituents at the $N^6$ position of the adenine ring. CK *de novo* biosynthesis is catalyzed by adenylate isopentenyl transferase (dimethylallyl-diphosphate:

*Abbreviations:* AtCKX, cytokinin dehydrogenase from *Arabidopsis thaliana*; BMS, *Zea mays* L., cultivar Black Mexican Sweet; CaMV35S, Cauliflower Mosaic Virus promoter; CK, cytokinin; CKX, cytokinin dehydrogenase; ER, endoplasmic reticulum; GFP, green fluorescent protein; iP, $N^6$-(Δ;$^2$-isopentenyl)adenine; *Ler*, *Arabidopsis thaliana* ecotype Landsberg *erecta*; PEG, polyethylene glycol; YFP, yellow fluorescent protein; ZmCKX, cytokinin dehydrogenase from *Zea mays*.

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AMP dimethylallyltransferase; EC 2.5.1.27). Substrates of this reaction are adenosine di- or triphosphates and the side chain precursor dimethylallyl pyrophosphate. The reaction produces isopentenyladenine nucleotides (Kakimoto, 2001; Takei et al., 2001), which can be subsequently hydroxylated by CK-specific cytochrome P450 monooxygenase to form *trans*-zeatin nucleotides (Takei et al., 2004). On the other hand, *cis*-zeatin forms are produced by the degradation of prenylated tRNA (Miyawaki et al., 2006). CK free bases are released from the nucleotides by hydrolytic cleavage catalyzed by a specific phosphoribohydrolase (Kurakawa et al., 2007). CK free bases can be further glycosylated on the adenine ring or its side chain by several strictly specific glucosyltransferases and thus generate N-glucosides or O-glucosides, respectively (Bajguz and Piotrowska, 2009). Some of these forms are considered biologically inactive, but they may be reactivated by CK-specific glucosidases.

CKXs are flavoproteins catalyzing irreversible cleavage of side chain of the CK moiety. Final products of this catalytic reaction are adenine or its derivatives and aldehyde originating from the side chain. Notably, CKX enzymes can work in both oxidase as well as dehydrogenase mode (Galuszka et al., 2001). Thus, the final electron acceptors of the reaction are either molecular oxygen or quinone derivatives. Quinone electron acceptors are preferred over oxygen and the rate of dehydrogenase reaction is substantially higher compared to the oxidase mode for all CKXs tested to date, including the entire maize CKX family (Frébortová et al., 2010; Zalabák et al., 2014).

A plant genome usually comprises several genes encoding for CKX isozymes and forming a small gene family. Seven *AtCKX* genes have been identified and functionally characterized in an *Arabidopsis* model (Bilyeu et al., 2001; Werner et al., 2001, 2003; Köllmer et al., 2014). Other fully characterized CKX families include 11 members each of rice (Ashikari et al., 2005; Hirose et al., 2007) and barley genomes (Mameaux et al., 2012; Mrízová et al., 2013) and 13 *ZmCKX* genes in maize (Vyroubalová et al., 2009; Zalabák et al., 2014).

All CKX isozymes show differences in their kinetic parameters and especially in their preferences for CK substrates. Some isoforms prefer CK free bases while others prefer CK nucleotides or glucose conjugates. This phenomenon has been described thoroughly in both *Arabidopsis* (Galuszka et al., 2007; Kowalska et al., 2010) and maize (Šmehilová et al., 2009; Zalabák et al., 2014). CKX isoforms also show distinct spatial and temporal patterns of expression (Vyroubalová et al., 2009; Zalabák et al., 2014).

Probably the most interesting feature characterizing diversity in the CKX family is the distinct subcellular localization of individual isoforms. It was previously shown in *Arabidopsis* that only 1 out of 7 AtCKX isoforms is cytosolic (Köllmer et al., 2014) and 2 isoforms are vacuolar. All of the remaining isoforms are associated with endoplasmic reticulum (ER) and are most probably secreted to the apoplastic space (Werner et al., 2003). To date, the localization of two CKX isoforms has been revealed in *Zea mays* L. ZmCKX1 was shown to be the apoplastic form. This was confirmed by two independent approaches, immunolabeling and green fluorescent protein (GFP) tagging (Galuszka et al., 2005; Zalabák et al., 2014). In contrast, ZmCKX10 lacks any signal peptide sequence. This isoform is thus localized in the cytosol and partially in the nucleus (Šmehilová et al., 2009).

The current study broadens the scope of our previous research, which had presented a detailed biochemical characterization of the entire maize CKX family (Zalabák et al., 2014). Here, we aimed to decipher the subcellular localization of major ZmCKX isoforms and thus to fill in the gaps in our current knowledge as to the spatial distribution of CK catabolism in monocot plants.

## 2. Materials and methods

### 2.1. Antibody production and purification

Recombinant ZmCKX2 and ZmCKX10 proteins, prepared in previous study (Zalabák et al., 2014), were used as antigens for production of polyclonal rabbit anti-ZmCKX antibodies. The antibodies were produced by Moravian-Biotechnology, Ltd. (Czech Republic). First, IgG fractions were purified from rabbit sera using Protein A-agarose (Roche) following the manufacturer's protocol. To increase the specificity of binding, the antibodies were further cross-purified. For this purpose, ZmCKX2 and ZmCKX10 proteins were covalently coupled to cyanogen bromide-activated-Sepharose® 4B matrix (Sigma), according to manufacturer's protocol. The protein A-purified ZmCKX antibodies were loaded onto the Sepharose column containing the reciprocal form of ZmCKX isozyme, i.e. anti-ZmCKX2 antibody was purified on the ZmCKX10-Sepharose 4B affinity matrix, and vice versa. This way non-specific IgG fractions remained bound to the matrix, whereas the specific passed through and were collected and concentrated by ultrafiltration for further use.

In addition, four peptide-specific antibodies raised against ZmCKX1, ZmCKX4b, ZmCKX6 and ZmCKX10 were prepared by commercial polyclonal antibody service (GenScript, USA). The protein sequences of ZmCKXs were screened to find amino acid regions unique to individual ZmCKXs. Peptides consisting of 12–14 amino acids were used for rabbit immunization (Supplementary Table 1).

The specificity of polyclonal rabbit antibodies, raised against recombinant proteins as well as against specific peptides, was tested using western blot analysis on both recombinant ZmCKX proteins (Zalabák et al., 2014) as well as maize protein extracts.

### 2.2. Cloning of ZmCKX–GFP constructs

Open reading frames of ZmCKXs were amplified without a termination codon using the Pfu DNA polymerase (Fermentas) from cDNA prepared in our previous studies (Zalabák et al., 2014; Šmehilová et al., 2009). The forward and reverse primers were designed to contain SpeI and HindIII/AgeI/AvrII restriction sites (Supplementary Table 2), respectively. PCR reactions were performed in the presence of 5% dimethyl sulfoxide. PCR products were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid) and subcloned into pLNU–GFP vector (http://www.dna-cloning.com/vectors/Vectors_with_markers/pLNU-GFP.gb).

Similarly as described above, cDNA sequences were subcloned into the pENTR™2B dual selection vector (Invitrogen) using primers designed to contain SalI/KpnI and EcoRI restriction sites and forward primers designed to carry the Kozak sequence CCACC upstream of the initiation codon (Supplementary Table 2). Constructs ZmCKX2$\Delta N_{term}$(1–42) and ZmCKX9$\Delta N_{term}$(1–43) were cloned without putative N-terminal sequence. The entry clones were recombined with the pGWB5 vector (Nakagawa et al., 2007), allowing for C-terminal GFP fusion, by LR recombination reaction (Gateway® technology, Invitrogen).

Constructs for stable maize transformation were prepared as described in Mohanty et al (Mohanty et al., 2009a, 2009b). Briefly, primers P1 and P2 were used to amplify the 5′ regulatory region and the coding region of the *ZmCKX2* gene, extending to the position where the yellow fluorescent protein (YFP) tag was inserted. The primer pair P3 and P4 was used to amplify the remainder of the *ZmCKX2* gene as well as the 3′ regulatory regions. Maize genomic DNA isolated from *Z. mays* L. cultivar B73 was used as a template to amplify both P1-P2 and P3-P4 fragments. The fragment carrying the citrine YFP coding sequence (citrine YFP-TT) was integrated in