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Research article

Biochemical characterization of the maize cytokinin dehydrogenase family and cytokinin profiling in developing maize plantlets in relation to the expression of cytokinin dehydrogenase genes

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ABSTRACT

The cytokinin dehydrogenases (CKX; EC 1.5.99.12) are a protein family that maintains the endogenous levels of cytokinins in plants by catalyzing their oxidative degradation. The CKX family in maize (*Zea mays* L.) has thirteen members, only two of which - ZmCKX1 and ZmCKX10 - have previously been characterized in detail. In this study, nine further maize CKX isoforms were heterologously expressed in *Escherichia coli*, purified by affinity and ion-exchange chromatography and biochemically characterized. ZmCKX6 and ZmCKX9 could only be expressed successfully after the removal of putative sequence-specific vacuolar sorting signals (LLPT and LPTS, respectively), suggesting that these proteins are localized to the vacuole. Substrate specificity analyses revealed that the CKX isoforms can be grouped into two subfamilies: members of the first strongly prefer cytokinin free bases while members of the second degrade a broad range of substrates. The most active isoform was found to be ZmCKX1. One of the studied isoforms, ZmCKX6, seemed to encode a nonfunctional enzyme due to a mutation in a conserved HFG protein domain at the C-terminus. Site-directed mutagenesis experiments revealed that this domain is essential for CKX activity. The roles of the maize CKX enzymes in the development of maize seedlings during the two weeks immediately after radicle emergence were also investigated. It appears that ZmCKX1 is a key regulator of active cytokinin levels in developing maize roots. However, the expression of individual CKX isoforms in the shoots varied and none of them seemed to have strong effects on the cytokinin pool.

1. Introduction

Cytokinins are ubiquitous plant hormones that govern a wide range of plant developmental and physiological processes. Cytokinin homeostasis in plant tissues and organs is crucial for normal growth and development, so cytokinin levels within cells, tissues, and organs must be finely controlled. One of the most important mechanisms by which this is achieved involves the irreversible degradation of active cytokinins by cytokinin dehydrogenases.

Cytokinins are chemical compounds that derive from adenine. The adenine moiety is substituted with either isoprenoid or aromatic side chains at the N^6- position to form so-called cytokinin free bases. *In planta*, cytokinins are synthesized from adenosine mono-, di- and triphosphates and dimethylallyl pyrophosphate (DMAPP), which functions as a side-chain precursor. This biosynthetic process is

Abbreviations: AtCKX, cytokinin dehydrogenase from *Arabidopsis thaliana*; CKX, cytokinin dehydrogenase; cZ, *cis*-zeatin; cZ9G, *cis*-zeatin-N9-glucoside; cZRMP, *cis*-zeatin riboside-5′-monophosphate; DCPIP, 2,6-dichlorophenolindophenol; DHZ, dihydrozeatin; DMAPP, dimethylallyl diphosphate; EDTA, ethylenediaminetetraacetic acid; iP, N^6-(Δ^2-isopentenyl)adenine; iP9G, N^6-(Δ^2-isopentenyl)adenine-N9-glucoside; iPR, N^6-(Δ^2-isopentenyl)adenosine; iPRMP, N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate; IPT, isopentenyl transferase; tZ, *trans*-zeatin; tZ9G, *trans*-zeatin-N9-glucoside; tZR, *trans*-zeatin riboside; tZRMP, *trans*-zeatin riboside-5′-monophosphate; ZmCKX, cytokinin dehydrogenase from *Zea mays*.

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catalyzed by isopentenyl transferase (IPT; dimethylallyldiphosphate: AMP dimethylallyltransferase; EC 2.5.1.27) enzymes and yields isopentenyladenine nucleotides (Kakimoto, 2001; Takei et al., 2001), which can be further hydroxylated by cytokinin-specific cytochrome P450 monooxygenase to form *trans*-zeatin nucleotides (Takei et al., 2004). Nucleotides of both isopentenyladenine and *trans*-zeatin are activated by cytokinin-specific phosphoribohydrolases to form free bases (Kurakawa et al., 2007), which can be further modified, mainly by glycosylation of the adenine moiety or the zeatin side chain forming N-glucosides and O-glucosides, respectively (reviewed by Frébortet al. 2011).

Cytokinin dehydrogenases (CKX; N6-dimethylallyladenine: acceptor oxidoreductase; EC 1.5.99.12) are flavoproteins that catalyze the irreversible cleavage of the side chains from cytokinin molecules. The final products of these cytokinin degradation reactions are adenine derivatives and aldehydes formed from the side chains. It was originally assumed that these enzymes function exclusively as oxidases, with molecular oxygen serving as the final electron acceptor (Pačes et al., 1971). However, they were subsequently reclassified as dehydrogenases because it was found that they strongly prefer electron acceptors such as quinones rather than molecular oxygen (Galuszka et al., 2001; Frébortová et al., 2010).

The cytokinin dehydrogenases are usually encoded by a small family of genes. The number of genes within these families varies from species to species: the *Arabidopsis thaliana* genome has seven, but monocot species tend to have more. For example, rice and *Brachypodium* have 11, and maize has 13 (Mameaux et al., 2012).

Even though they catalyze similar reactions, different CKXs have different rates of reaction and substrate preferences (Galuszka et al., 2007; Kowalska et al., 2010). They also usually have different spatial and temporal expression patterns. That is to say, different CKX enzymes are expressed in different tissues and organs, and at different stages of development. It has been shown that the levels of *CKX* gene expression can change very rapidly and dramatically, especially in response to stress signals (Vyroubalová et al., 2009). Because each CKX enzyme has unique substrate preferences and reaction rates, localized changes in the expression of specific *CKX* genes can cause pronounced changes in the cytokinin distribution within individual tissues and organs. This in turn affects their growth and development. The different CKX enzymes are also targeted to different subcellular compartments. Of the seven *Arabidopsis* CKX isoforms, one is cytosolic, two are vacuolar, and the others are secreted into the extracellular environment. Moreover, CKX overexpression studies have suggested a close correlation between the subcellular localization of the overexpressed CKX enzyme and the severity of the resulting cytokinin deficiency phenotype (Werner et al., 2003). One maize CKX isoform, ZmCKX1, is known to be localized to the apoplast while another, ZmCKX10, is cytosolic (Šmehilová et al., 2009). While the localization of the remaining maize CKX isoforms is currently unknown, it can reasonably be expected to differ somewhat from that of their counterparts in *Arabidopsis*, especially given the recently reported differences in cytokinin metabolite levels between monocots and dicots (Gajdošová et al., 2011).

In the work reported herein, we characterized the entire maize *CKX* gene family in detail in order to understand how each member contributes to the regulation of cytokinin homeostasis. Maize was selected because it is an important model species for monocots in general. This work builds on our previous investigations into its CKX enzymes (Vyroubalová et al., 2009; Šmehilová et al., 2009), which focused on the roles of cytokinin metabolism in abiotic stress responses and the subcellular localization of two maize CKX isoforms (ZmCKX1 and ZmCKX10), respectively.

2. Results and discussion

2.1. Heterologous expression and purification of ZmCKXs

ZmCKX proteins were prepared by means of intracellular heterologous expression in *Escherichia coli* using the pTYB12 expression vector, which enables quick and easy affinity purification of the inserted protein because it fuses a self-cleavable chitin-binding intein tag to the protein's N-terminus. The sole exception was the ZmCKX1 enzyme, which was prepared in a *Pichia pastoris* expression system (Bilyeu et al., 2001), as the recombinant enzyme obtained by expression of *ZmCKX1* gene in yeast was used in most biochemical studies of ZmCKX1 performed up to date and thus served as a reference.

As shown in *Arabidopsis*, the various CKX isoforms encoded in the genome of an individual species have distinct biochemical properties, are expressed in different tissues, and have different subcellular localizations (Galuszka et al., 2007; Werner et al., 2003). The localization of each isoform is determined by an N-terminal sequence motif. In previous experiments, the presence of these signal sequences was found to impair the heterologous expression of some *Arabidopsis* CKX isoforms in *P. pastoris*; this effect could be alleviated by using expression vectors containing cloned CKX genes in which the targeting sequences had been removed (Kowalska et al., 2010). These sequences can also cause undesirable interactions between heterologous proteins and the post-translational processing machinery of *E. coli*, resulting in the formation of non-functional heterologous proteins and/or their targeting to inclusion bodies. The predictive tool SignalP 4.0 (Petersen et al., 2011) was therefore used to identify sequences encoding potential N-terminal signal peptides in the ZmCKX proteins. SignalP 4.0 was unable to identify the signal peptide sequence of ZmCKX9 so a different predictive tool, iPSORT, was used in this case (Bannai et al., 2002). Putative N-terminal signal sequences were found in all of the studied sequences (namely ZmCKX1; 2; 3; 4a; 4b; 5; 6; 8; 9 and 12) with the exception of the cytosolic ZmCKX10 (Fig. 1). The signal sequences of enzymes ZmCKX1 to 6, ZmCKX9, ZmCKX11 and ZmCKX12 seem to direct their secretion to the apoplast whereas that of ZmCKX8 was predicted to be a mitochondrial targeting sequence. However, there is no precedent for CKX proteins being localized in the mitochondria, so it seems likely that this is a case of misidentification. Notably, two *Arabidopsis* CKX isoforms, AtCKX1 and AtCKX3, were initially predicted to be localized to the mitochondria (Schmülling et al., 2003). However, subsequent GFP tagging experiments demonstrated that they are actually localized to the vacuoles (Werner et al., 2003).

The removal of the putative secretory signals strengthened the expression of some of the recombinant CKX proteins, thus all of them were cloned without them. For example, clarified extracts of bacteria expressing the entire *ZmCKX12* ORF exhibited negligible cytokinin degradation activity. However, significant activity was detected in the equivalent extracts of bacteria expressing the same gene with the signal peptide removed. Therefore, the secretory signals were removed from all of the cloned *CKX* genes that were inserted into the expression vectors.

There are several closely homologous *CKX* gene pairs in the maize genome that were formed during recent chromosome duplication events (Gaut, 2001). The enzymes ZmCKX7 and ZmCKX11 are very close paralogs of ZmCKX8 and ZmCKX12, respectively, having very high levels of amino acid sequence identity (96.7 and 91.5%, respectively), and were excluded from this study because they are very weakly expressed in maize, and their abundance was below the limit of detection for the method used in this work (Vyroubalová et al., 2009). All four of the recombinant proteins corresponding to two other closely homologous pairs