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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 ZmCKX2ΔN_{term}(1–42) and ZmCKX9ΔN_{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

between P1-P2 and P3-P4 fragments using the triple-template PCR method. The final P1-P2-citrine YFP-TT-P3-P4 fragment was subcloned in pDONR207 vector using BP reaction and later using LR reaction (Gateway® technology, Invitrogen) to pAM1006 binary vector.

2.3. Maize suspension cell culture cultivation, isolation and protoplast transfection

Cell suspension culture of *Z. mays* L., cultivar Black Mexican Sweet (BMS) was kindly provided by Dr. François Chaumont and Dr. Nicolas Richet from the Katholieke Universiteit Leuven (Belgium). Cell culture was cultivated in BMS medium (Murashige–Skoog medium, pH 5.7, supplemented with 3% sucrose, 0.2 g L^{-1} L-asparagine, and 13.5 μM 2,4-dichlorophenoxyethanoic acid). The cell culture was grown in 250 mL Erlenmeyer flasks on a rotary shaker (100 rpm) at 23 °C in darkness. The culture was passaged every 10 days by inoculating 10 mL of culture into 40 mL of fresh BMS medium. Plasmid constructs ZmCKX–pLNU–GFP were co-transfected together with the CaMV35S:AtWAK2-HDEL-tdTomato construct vector (ER-tdTomato) into maize protoplasts using the polyethylene glycol method. Construct ER-tdTomato, kindly provided by Dr. Dirk Becker (University of Hamburg), encodes for ER marker peptide fused with red fluorescent tag protein tdTomato (tandem dimer Tomato). The ER marker was created by combining the signal peptide of AtWAK2 (*Arabidopsis thaliana* wall-associated kinase 2 (He et al., 1999);) at the N-terminus of the fusion protein and the ER retention signal His-Asp-Glu-Leu at its C-terminus (HDEL (Gomord et al., 1997),). Protoplasts were released from cultured cells using 1.5% Cellulase Y-G and 0.3% Macerozyme R10 (Serva) in digestion buffer (0.65 M D-sorbitol, 10 mM KCl, 1 mM $CaCl_2$, 8.7 mM MES, pH 5.5). Protoplasts were filtered through 70 μm nylon mesh and then washed twice with digestion buffer without enzymes. Protoplasts (about 10,000) were resuspended in 100 μL of transfection medium (0.6 M mannitol, 8.5 mM $CaCl_2$, 5 mM MES, pH 5.7) and 10–15 μg of each plasmid was added. The suspension was then mixed with 110 μL of PEG medium (40% PEG 4000, 0.1 M $Ca(NO_3)_2$, 0.3 M mannitol) and incubated for 2 min at room temperature. After the addition of 440 μL of W5 medium (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 4 mM MES, pH 5.7), the protoplasts were collected by centrifugation at 100 *g* for 1 min, resuspended in 1 mL of digestion buffer without enzymes, then held in darkness at room temperature for approximately 20 h.

2.4. Arabidopsis thaliana Ler suspension cell culture transformation

The ZmCKX-pGWB5 constructs were introduced into *Agrobacterium tumefaciens* cells of the GV3101 strain by electroporation. Cell suspension culture of *A. thaliana* ecotype Landsberg *erecta* (*Ler*) was kindly provided by Dr. Beata Petrovská from IEB AS CR in Olomouc, Czech Republic. Culture was cultivated in LER medium (Murashige–Skoog medium, pH 5.7, supplemented with 3% sucrose, 550 nM 2-(1-naphthyl)acetic acid and 250 nM kinetin). *Arabidopsis* L*er* cell cultures were maintained in the same way as were maize suspension cultures. The suspension culture was transformed with *A. tumefaciens* GV3101, carrying ZmCKX-pGWB5 plasmid constructs, according to the protocol adapted from Mathur et al (Mathur et al., 1998). Briefly, *Agrobacterium* starter culture in LB media containing appropriate antibiotics (25 mg L^{-1} gentamycin, 25 mg L^{-1} rifampicin, and 50 mg L^{-1} kanamycin) was grown at 28 °C until OD_{600} reached 0.5. The culture was pelleted at 4000 *g* and resuspended in 1 mL of LER media supplemented with 50 μM acetosyringone and cultivated at room temperature for 2.5 h. L*er* cultures 2 days old were transformed by adding 200–500 μL of bacterial inoculum into the culture. After 2 days of co-cultivation under standard cultivation conditions, the quality of L*er* cells and the presence of bacterial cells were examined under a microscope. When the bacteria had overgrown, L*er* cells were harvested by centrifugation at 700 *g* at room temperature and the medium was exchanged. This step was repeated every second day. After 2 weeks of cultivation, hygromycin B (25 mg mL^{-1}) was added to the L*er* culture to select the transformed *A. thaliana* cells. Timentin (250 mg L^{-1}) was added after 3–5 days to remove bacteria. The transgenic L*er* culture was usually stabilized 3–4 weeks after the transformation, and thus passaged once per week. The quality of the cells and presence of the fluorescence signal were regularly controlled under an epifluorescence microscope. To measure CKX activity, cells from cultures (50 mL) 5 days old and cultivated in the LER media buffered by 20 mM potassium phosphate buffer (pH 5.7) were harvested by filtering through a Buchner funnel. Proteins in the medium were concentrated by lyophilization and resuspended in the CKX activity buffer.

2.5. Generation of ZmCKX2-YFP maize transgenic lines

The construct ZmCKX2-YFP-pAM1006 was electroporated in *A. tumefaciens* strain EHA101 and selected on LB plates containing spectinomycin (100 mg L^{-1}), kanamycin (50 mg L^{-1}), and chloramphenicol (25 mg L^{-1}). *Agrobacterium*-mediated transformation of maize seedlings and subsequent screening of transgenic lines were performed as described by Mohanty et al (Mohanty et al., 2009a, 2009b).

2.6. Isolation of intact vacuoles

Intact vacuoles were isolated from 5-day-old *A. thaliana* L*er* suspension cells according to a protocol adapted from Robert et al (Robert et al., 2007). Briefly, 10 mL of culture were harvested by centrifugation at 500 *g*. The supernatant was removed and the cells were used for further protoplast isolation following the protocol.

2.7. Microscopic analysis of ZmCKX–GFP fusion protein

The transformed materials carrying ZmCKX–GFP fusions were mounted onto microscope slides, covered with a slip, and then studied under a Zeiss LSM710 laser scanning microscope with 20x objectives. GFP-tagged proteins and the ER-tdTomato marker protein were excited at laser wavelengths of 488 nm and 561 nm, respectively. Fluorescence emission was detected using a 505/530-nm band-pass filter for GFP and 570/590-nm band pass filter for ER-tdTomato. Image processing utilized Zeiss Zen software Black edition.

2.8. CKX activity assay

Plant material was frozen in liquid nitrogen immediately after harvesting. Protein extraction buffer (50 mM Tris/HCl, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, 100 nM bestatin, 1 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride PMSF, pH 8.0) was added in the ratio of 1 mL per 1 g of ground sample. The mixture was kept on ice and extracted for at least 20 min. After extraction, the sample was centrifuged at 20,000 *g* and 4 °C. Protein concentration in clarified extract and concentrated media was quantified using the Bradford method (Bradford, 1976). CKX activity in cell lysates and in culture media was measured by the end point method (Frébort et al., 2002). Aliquots of cell lysate (1–50 μL) or concentrated media (2–100 μL) were incubated at 37 °C for 2–16 h in a reaction mixture (total volume of 0.6 mL) consisting of 250 mM McIlvaine buffer (pH 7.5), 0.125 mM dichlorophenolindophenol, and 250 μM substrate iP.

2.9. Western blot analysis

Proteins were extracted from plant material as described in the previous paragraph. Maize extracts were prepared from two weeks old maize seedlings cultivar Cellux, cultivated hydroponically as described in (Zalabák et al., 2014). Maize reproduction organs (cobs and tassels) were collected in the field. SDS–PAGE was performed on a slab gel (10%) in Tris–glycine running buffer. The Precision Plus Protein™ unstained standard (Bio-Rad) was used as a marker. Protein samples were heated before application at 95 °C for 10 min in the presence of 2% SDS and 5% 2-mercaptoethanol. Proteins were blotted onto a PVDF membrane (0.45 μm, Millipore) in the Mini-Trans blot system (Bio-Rad). The membrane was stained with Amido black to visualize proteins. After decolorization in water, the membrane was blocked with 5% nonfat dried milk, 2% BSA in 20 mM Tris/HCl (pH 7.5), containing 500 mM NaCl (TBS buffer), for 90 min. After the blocking, the membrane was washed twice for 10 min in TBS buffer containing 0.05% of Tween-20 and then probed with primary rabbit antibody diluted in 1% nonfat dried milk, 0.4% BSA in Tween-TBS buffer (1:1000) for 90 min. ZmCKX-GFP fusion proteins were detected using polyclonal rabbit anti-GFP antibody ab290 (Abcam), diluted 1:1000. The membrane was subsequently rinsed twice with Tween-TBS and then incubated for 1 h with goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) in 1% nonfat dried milk, 0.4% BSA in Tween-TBS buffer (1:5000). After rinsing in Tween-TBS buffer, the membrane was developed using the Pierce ECL Western Blotting Substrate (Thermo Scientific) and recorded by system ChemiDoc MP (Bio-Rad).

2.10. Cytokinin analysis

CKs were purified from *A. thaliana* L*er* suspension cells as well as from isolated intact vacuoles and subsequently analyzed according to the procedure described in detail by Vyroubalová et al (Vyroubalová et al., 2009).

3. Results and discussion

3.1. Prediction of ZmCKX intracellular targeting and rationale for the localization studies

First, detailed *in silico* analysis of all ZmCKX protein sequences was performed to reveal possible protein sorting signals and obtain information about their putative distribution within the cell compartments (Table 1). The predictions showed that all ZmCKX isoforms, with the exception of ZmCKX8, ZmCKX9, and ZmCKX10, carried putative N-terminal secretory peptide. While the localization of ZmCKX1 to the apoplast had been clearly demonstrated using two diverse methods (Galuszka et al., 2005; Šmehilová et al., 2009), the subcellular localization of other signal peptide-containing isoforms remained unclear. The presence and proper function of N-terminal signal sequences within the ZmCKX proteins was clearly confirmed in our heterologous expression experiments. In this case, the presence of N-terminal signals impaired the protein expression in both *E. coli* as well as *Pichia pastoris*. In contrast, recombinant truncated forms without the signal sequences (mature proteins) were robustly expressed in both heterologous expression systems (Bilyeu et al., 2001; Zalabák et al., 2014).

Despite the unambiguous localization pattern of ZmCKX1, analysis of its protein sequence using the TargetP 1.1 tool was not able to detect the presence of any N-terminal signal sequences and showed only a very weak degree of reliability (reliability class [RC] 5).

On the other hand, ZmCKX10 lacks any signal peptide sequence and it was therefore predicted to be the cytosolic form, as previously confirmed experimentally (Šmehilová et al., 2009). Therefore, we used ZmCKX10 as a proof of concept in our experimental setup. In contrast, ZmCKX8 may contain a secretion peptide and/or mitochondrial targeting peptide, but the prediction's reliability was again weak (RC 5). ZmCKX9 may also possess a secretion peptide sequence and, in addition, a putative chloroplast transit peptide sequence was predicted at the protein N-terminus. Our previous study had demonstrated the presence of putative vacuolar retention signals LPTS and LLPT in the N-terminal part of ZmCKX9 and ZmCKX6, respectively (Zalabák et al., 2014). The presence of these signals also impaired successful heterologous expression in *E. coli*. Even though the LLPT signal was removed and (such a ZmCKX6) the mutant form was successfully expressed, the enzyme remained fully inactive. Interestingly, we obtained the active enzyme form of ZmCKX6 protein by the reverse mutation of phenylalanine in the conserved HFG motif (Zalabák et al., 2014).

ZmCKX7 and ZmCKX11, (very homologous duplicated) paralogs of ZmCKX8 and ZmCKX12, were not included in the localization study due to their extremely low expression levels in maize tissues (Vyroubalová et al., 2009). The other isoforms, ZmCKX3 and ZmCKX4b, share 96.2% and 94.5% similarity of amino acid sequence with ZmCKX2 and ZmCKX4a, respectively, and their biochemical properties are similar (Zalabák et al., 2014). To simplify workflow in time-consuming transformation experiments, the former multiplied isozymes(, as a result of a recent chromosomal duplication event) were excluded from the study because similar results can be

Table 1
In silico analysis of putative N-terminal signal peptides in maize CKX proteins. Three online prediction tools – SignalP 4.1 (Petersen et al., 2011), TargetP 1.1 (Emanuelsson et al., 2000), and iPSORT (Bannai et al., 2002) – were used for identification of putative sorting signals. TPlen indicates the length of putative signal peptide. D-score is used to discriminate signal peptides from non-signal peptides. For non-secretory proteins, all D-scores should ideally be very low (close to the negative target value of 0.1), while for secreted ones the score should exceed 0.45. RC stands for reliability class and ranges from 1 to 5, where 1 indicates the strongest prediction. Tested proteins can carry a secretion peptide (SP), a mitochondrial targeting peptide (mTP), a chloroplast transit peptide (cTP), and/or in the case of cytosolic proteins no signal peptide (–). To be judged as SP the score* must exceed the value 0.9225.

Enzyme	SignalP 4.1			TargetP 1.1			iPSORT	
	TPlen	D-score	SP	TPlen	RC	Localization	Score*	Localization
ZmCKX1	18	0.815	YES	18	5	SP	1.42	SP
ZmCKX2	24	0.772	YES	24	1	SP	1.725	SP
ZmCKX4a	21	0.863	YES	21	1	SP	1.715	SP
ZmCKX5	21	0.892	YES	21	1	SP	1.27	SP
ZmCKX6	23	0.595	YES	23	3	SP	1.42	SP
ZmCKX8	23	0.878	YES	86	5	mTP	1.455	SP
ZmCKX9	42	0.274	NO	42	4	SP	0.47	cTP
ZmCKX10	54	0.155	NO	–	2	–	−0.975	–
ZmCKX12	26	0.506	YES	26	2	SP	1.65	SP

anticipated.

To sum up, based on our former results, we selected 9 out of 13 maize CKX enzymes for investigation of their subcellular localization. For this purpose, we first performed the protein localization prediction analyses using three online tools. Our results suggest that the reliability of these prediction analyses on monocotyledonous proteins is generally low and these data must be verified experimentally.

3.2. Immunolocalization of ZmCKX isozymes

As described above, ZmCKX1 isozyme was proved to be localized in the apoplastic space in maize kernels using immunogold labeling method (Galuszka et al., 2005). We have decided to utilize a similar approach to localize further members of ZmCKX family in maize tissues. For this purpose, two polyclonal antibodies against ZmCKX2 and ZmCKX10 proteins were prepared and extensively purified to increase their specificity. Despite the fact that the specificity increased dramatically after this purification step, we could not detect any protein in extracts from selected maize tissues (Supplementary Fig. 1.). Further, we prepared four peptide-specific antibodies designed to recognize ZmCKX1, ZmCKX4b, ZmCKX6 and ZmCKX10 isozymes. While the specificity of these antibodies was excellent on recombinant ZmCKX proteins, the antibodies had not been active in our Western blot experiments with the maize tissue extracts probably due to the transient nature of ZmCKX isoforms expression in plant tissues/they again failed to detect their specific targets in the maize tissue extracts (data not shown).

Our experience with laborious antibody production and purification shows the difficulty to prepare the antibodies that would combine high specificity with strong avidity. This is due to high homology among the ZmCKX family members limiting the number of potential antigenic sites. In addition, with exception of ZmCKX1 the other isoforms of this family are present in minute amounts in maize tissues, which makes the detection very complicated. Therefore, we adopted the classical approach of GFP-tagging to study the subcellular localization of the fleeting ZmCKX isoforms.

3.3. Transient expression of ZmCKX isoforms in maize protoplasts

To follow the subcellular trafficking of maize CKX isozymes, the respective GFP-fused genes were transiently expressed in maize protoplasts derived from BMS suspension cell culture under the control of a strong ubiquitin 1 promoter (Fig. 1). The fusion of the GFP protein to the C-terminus of ZmCKX was selected to avoid mislocalization of the fusion proteins due to a possible overlap with the putative signal peptide sequences at the N-terminus of ZmCKX proteins. Prepared ZmCKX–pLNU–GFP constructs were co-transformed together with the CaMV35S:AtWAK2-HDEL-tdTomato vector, which we used as a marker for ER. Prepared constructs and the ER marker were introduced into maize protoplasts using the PEG-mediated transfer. Transient expression of the co-transformed cells was observed using a laser-scanning confocal microscope 20–24 h after the transformation.

The construct Ubi:GFP (free GFP) was selected as a control for transformations and was also co-transformed with ER-tdTomato marker/construct. The localization pattern of GFP transiently expressed in maize protoplasts partially coincided with the red signal of the ER marker protein (Fig. 1B). The overlap of the two signals was not complete, however, and free GFP signal prevailed. This is in accordance with the general view that cytosolic localization is typical for free GFP and, due to its small molecular mass, it can easily diffuse through the nuclear pores and accumulate in the nucleus (labeled with an asterisk in Fig. 1B).

Previous experiments have shown that ZmCKX1 localizes to the cell apoplast, which is to say that after its *de novo* synthesis ZmCKX1 enters the ER secretory pathway and is secreted to the extracellular space (Šmehilová et al., 2009). The transient expression of ZmCKX1–GFP in maize protoplasts revealed a mesh-like pattern, which is characteristic of ER (Fig. 1B). Co-localization with the ER marker further showed a complete overlap of the two signals, strongly suggesting that ZmCKX1 was localized to ER structures several hours after the transformation. Based on the same targeting prediction (Table 1), similar results were anticipated in the cases of ZmCKX2, ZmCKX4a, ZmCKX5, ZmCKX6, and ZmCKX12. Indeed, the distribution patterns of these ZmCKX–GFP fusions were similar to that of ZmCKX1–GFP, further supporting the view that these isoforms are localized to the ER and later perhaps secreted (for details see Fig. 1B and C, respectively). A similar localization pattern was observed also for ZmCKX8, despite the ambiguous results coming from the prediction analysis for this isoform (Fig 1C). While *ZmCKX6* encodes for a nonfunctional enzyme, our results from BMS transformations showed that the enzyme can be transiently expressed (Fig. 1B) with localization to the ER. In the case of ZmCKX2–GFP and ZmCKX8–GFP, the fluorescent signal was also confined to vesicular structures reminiscent of putative prevacuolar compartments (see Fig. 1B and C, indicated with arrowheads). The localization of these ZmCKX isoforms that were previously speculated to be present primarily in the cell apoplast should, however, be interpreted in the context of the expression system that was used. The transient assay that we used (i.e., maize BMS protoplast assay) is an established system for studying protein localization. Particle bombardment and PEG-mediated protoplast transformation constitute the most common methods for transient protein expression. Due to the fact that stable transformation of monocots is a laborious and time-consuming process, transient expression systems are often the method of choice for protein localization studies. It is possible, however, that proteins subjected to intracellular sorting pathways may respond sensitively to the specific nature of these undifferentiated cells and the relatively short period of the protein expression. Hence, we might observe the initial phase of proteosynthesis and processing of strongly overexpressed ZmCKXs occurring on ribosomes and structures of the ER. In addition, it should be noted that no putative ER retention motifs (e.g., K/HDEL signal) are present in the sequence of any ZmCKX enzymes.

In the case of ZmCKX9, information on its putative targeting was contradictory (see Table 1). Moreover, our earlier results had revealed the presence of a putative N-terminal vacuolar retention signal (Zalabák et al., 2014). Based on this, the signal of the ZmCKX9–GFP fusion transiently expressed in maize protoplasts was expected to be localized to vacuoles. In contrast, no vacuolar targeting of the isoform was in fact observed and all of the transformed cells again showed characteristic ER distribution of the signal (Fig. 1C).

Further, the signal of the ZmCKX10–GFP fusion was found to be only partially overlapping with the red fluorescent signal of ER-tdTomato in the co-transformed maize protoplasts and the ZmCKX10–GFP localization was suggestive of the cytosol and nuclei (see Fig. 1C, nucleus indicated by an asterisk). These results are in good agreement with our current knowledge regarding this enzyme, as there is no signal peptide for intracellular sorting. As shown previously, the stable expression of the ZmCKX10–GFP fusion in tomato hairy roots yields cytoplasmic and nuclear localization (Šmehilová et al., 2009).

An obvious benefit of using the transient maize expression assay is the fact that this system allows for the homologous expression. Therefore, the studied maize proteins are expressed in their natural environment and their signal peptides, retention signals, and other protein motifs should be correctly recognized and interpreted. It

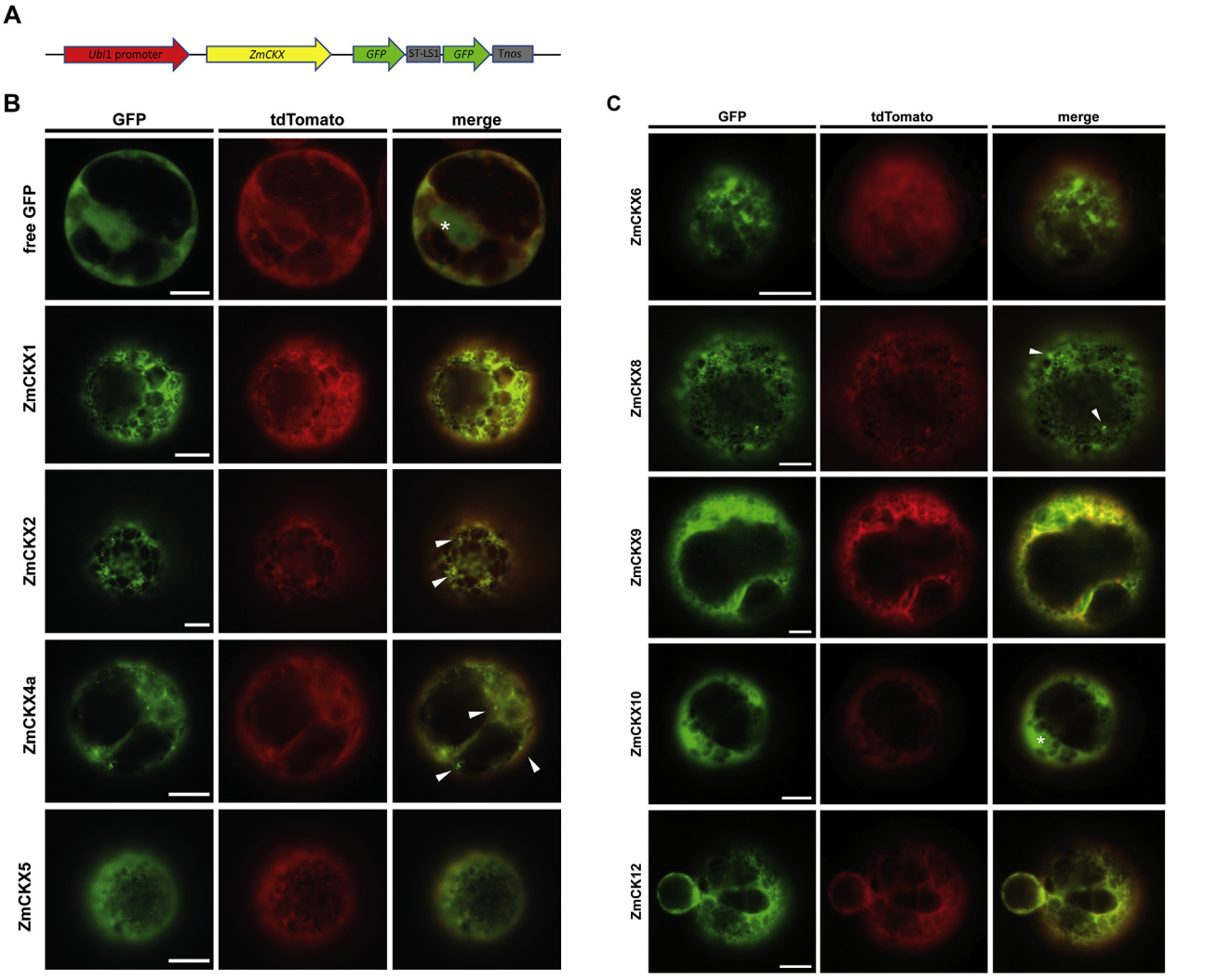


Fig. 1. Transient expression of ZmCKX–GFP fusion proteins in maize protoplasts. (A) Schematic representation of pUbi1:ZmCKX–GFP constructs used for transient transformation. Constructs carry maize *Ubi*1 promoter, maize *ZmCKX*, *GFP* with ST-LS1 intron and T*nos* terminator sequences. Protoplasts transiently co-transformed with plasmid constructs pUbi1:ZmCKX-GFP and CaMV35S:AtWAK2-HDEL-tdTomato (a marker for ER) were examined under a scanning confocal microscope after 20 h incubation (B, C). Free GFP constitutes a control transformed by Ubi1:GFP construct. Green channel (denoted as GFP, left column) showing free GFP and ZmCKX-GFP signal and red channel showing ER marker tdTomato (middle column) were captured and then merged (right column). Co-localization of GFP signal with ER is represented by yellow color. Asterisk indicates nucleus. Arrowheads indicate putative prevacuolar compartments. Scale bars represent 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

should be acknowledged that the constitutive expression of the tested genes, driven by ubiquitin 1 promoter, is non-physiological inasmuch as the protein is overproduced, and thus artifacts can be observed as over-accumulation on ER structures. Nevertheless, the ubiquitin 1 promoter is derived from the maize genome (Christensen et al., 1992) and therefore drives homologous expression. Moreover, the proper localization of ZmCKX10–GFP and free GFP in the cytosol and the ER-tdTomato marker protein in the ER suggests that the expression system utilized was working properly and the results are thus correct and reproducible.

3.4. Stable expression of ZmCKX–GFP fusions in A. thaliana Ler suspension cells

The transient expression of GFP-fused CKX enzymes brought new evidence on the subcellular pattern of distribution in maize protoplasts. First of all, predominant ER localization of the majority of ZmCKX isoforms was observed, even though we could identify no ER retention signal in their protein sequences. It is therefore possible that these isoforms might be further sorted within the cell in a time-dependent manner. To gain more insight into the localization pattern of ZmCKX isoforms in stably expressing cells, we adopted *Arabidopsis* cell culture assay with *Agrobacterium*-mediated transformation.

Notably, ZmCKX1–GFP showed only a weak and unspecific fluorescent signal in confocal microscopy observations with no apparent accumulation of the signal (data not shown). In contrast, expression of the protein in the maize protoplasts observed 1 day after the transformation revealed an association of ZmCKX1–GFP with ER structures (Fig. 1B). This leads us to assume that the transient maize protoplast assay shows only the initial phase of ZmCKX1 trafficking in the cell sorting system. As we have shown previously, the N-terminal secretion peptide which is present in the ZmCKX1 protein sequence drives its secretion to the apoplastic space in maize kernel tissues (Galuszka et al., 2005). As plant tissues are compact and plant cells hold together via cell walls, the secreted ZmCKX1 is retained in the apoplast. Conversely, the *Arabidopsis Ler* culture consists of individual cells which do not form an

Table 2
CKX activity in ZmCKX–GFP suspension cultures of *Arabidopsis*. Specific activities were determined in 250 mM McIlvaine buffer (pH 7.5), with 250 μM iP as a substrate and 0.125 mM dichlorophenolindophenol as an electron acceptor. Asterisks denote lines in which GFP signal vanished during selection. Data for recombinant ZmCKXs are extracted from Zalabák et al (Zalabák et al., 2014). ND – value below the limit of detection.

Specific activity (pkat mg^{-1} of protein)					nkat mg^{-1}
Source	Media		Lysate		Recombinant enzyme
	Value	SE	Value	SE	Value
WT	1.38	0.54	0.04	0.033	–
GFP	1.74	0.99	0.08	0.02	–
ZmCKX1-GFP	408.37	43.61	2.11	0.10	890.38
ZmCKX2-GFP	2.78	0.28	0.75	0.11	29.29
ZmCKX4a-GFP	1.73	0.93	0.37	0.21	13.90
ZmCKX5-GFP	5.54	2.15	3.10	0.22	42.87
ZmCKX6-GFP*	1.93	0.39	0.20	0.14	ND
ZmCKX8-GFP	2.31	0.41	2.18	0.23	51.23
ZmCKX9-GFP	8.53	1.83	1.16	0.01	0.64
ZmCKX10-GFP*	1.66	0.19	0.16	0.02	9.21
ZmCKX12-GFP	2.40	0.19	1.32	0.02	26.83

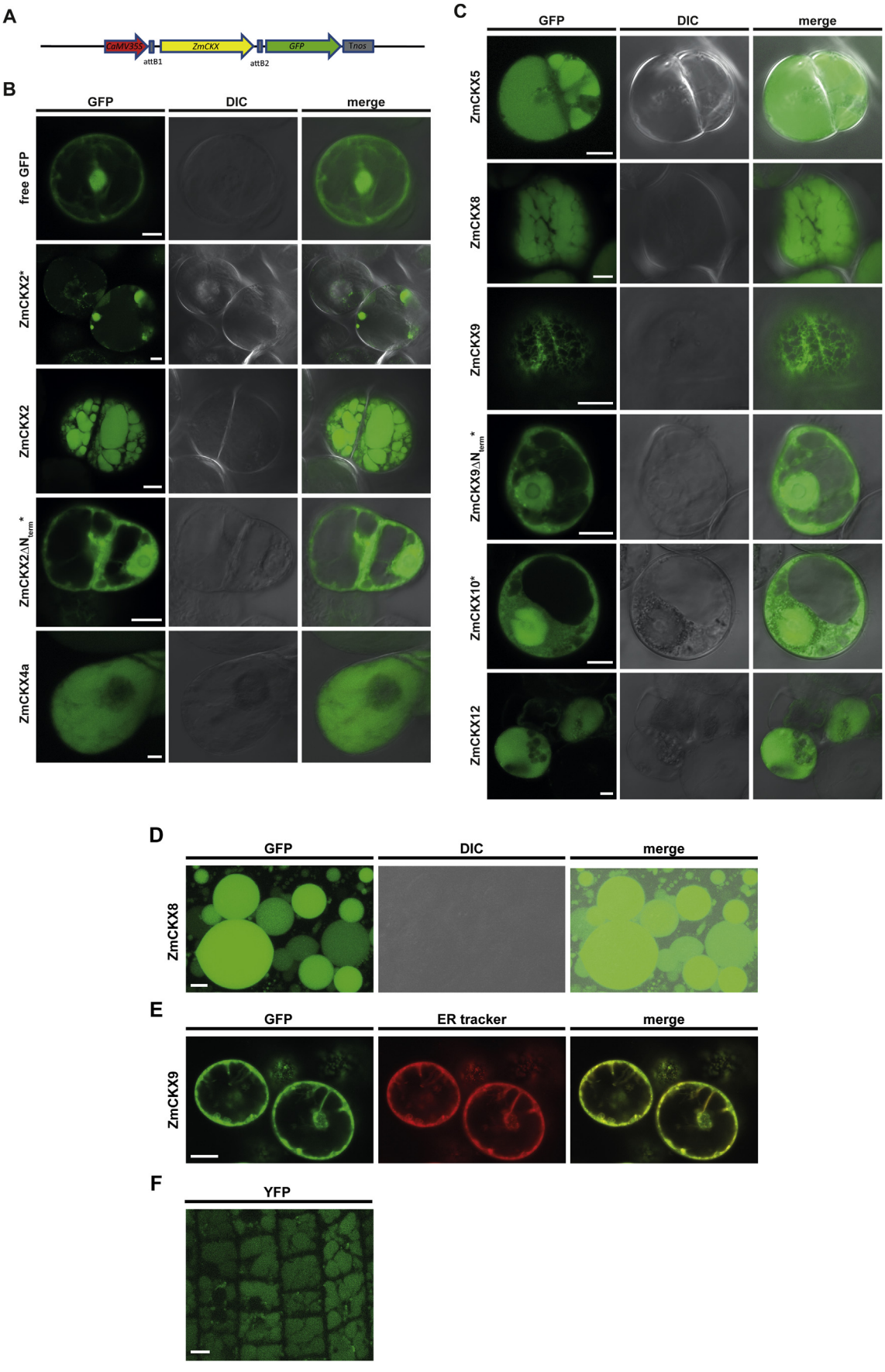
apoplast and therefore soluble proteins, such as ZmCKX1, can be washed out of the cell wall. To prove the hypothesis, CKX activity was measured in the culture medium as well as in the crude cell extract of the ZmCKX1–GFP cell line (Table 2). As expected, only marginal CKX activity was recorded in the culture media of the control lines (WT and free GFP cell lines; Table 2). This background activity most probably corresponds to the endogenous activity of secreted *Arabidopsis* CKX isoforms (Werner et al., 2003). In contrast to these results, the CKX activity in the culture media of the ZmCKX1–GFP expressing cell line increased several hundred times and the intracellular CKX activity in the cell extracts increased approximately 50-fold compared to the control. These results clearly show that ZmCKX1 is secreted primarily outside the cell, and that is in agreement with our previous findings (Galuszka et al., 2005; Šmehilová et al., 2009). In addition, no signal corresponding to ZmCKX1-GFP fusion protein was detected using anti-GFP antibody in western blot experiment (Supplementary Fig. 2). As ZmCKX1 is a soluble protein and localizes to the apoplastic space, it can be easily transported throughout the plant tissues and organs. High CKX activity had also been recorded previously in xylem exudates of young maize seedlings (Podlešáková et al., 2012). The apoplastic transport of this isoform together with its extremely high reaction rate (Zalabák et al., 2014) and prompt CK-inducible activation (Vyroubalová et al., 2009) make ZmCKX1 the main tool for rapid and effective regulation of CK homeostasis in maize tissues and organs.

As noted above, the ZmCKX2 isoform was also predicted to be targeted to the apoplast. However, the stable expression of GFP fusion protein in *A. thaliana* L*er* suspension cells showed that ZmCKX2–GFP was primarily localized in the vacuoles. In the freshly transformed culture (about 2 weeks after transformation), the fluorescent signal was observed in small vesicular structures reminiscent of prevacuolar compartments (Fig. 2B, designated with asterisk). Later, 4 weeks after the transformation, these structures fused and large vacuoles filling most of the cells' inner space were observed (Fig. 2B). While no increase in CKX activity was detected in the cultivation media, the activity in the crude cell extract was 18-fold higher in comparison to that in the WT cell line (Table 2). These results together with the result of western blot analysis (Supplementary Fig. 2.) confirm that ZmCKX2 is not secreted but is indeed retained within the cell. To prove that vacuolar targeting of ZmCKX2 is due to the N-terminally localized signal peptide sequence, the construct lacking the corresponding region was prepared and expressed in *A. thaliana* L*er* suspension cells. In contrast to the situation described above, the GFP signal in ZmCKX2ΔN_{term}(1–42)-GFP mutant was distributed mainly in the cytosol and nucleus (Fig. 2B). This is in complete agreement with the localization pattern of the signal peptide-lacking isoform ZmCKX10, which clearly demonstrates that the N-terminal signal peptide is important for vacuolar targeting of ZmCKX2.

A vacuolar localization pattern similar to that seen in the case of ZmCKX2–GFP was observed for ZmCKX4a–GFP, ZmCKX5–GFP, ZmCKX8–GFP, and ZmCKX12–GFP fusions. CKX activity was measured in the cell lysates and culture media for these isoforms to confirm the functionality and the exclusively intracellular localization. ZmCKX4a–GFP, ZmCKX8–GFP, and ZmCKX12–GFP showed only background CKX activity in the culture media comparable to that of the control cell lines. On the other hand, the intracellular CKX activity in these lines increased 33- to 54-fold when compared to the WT control. Although only a 4-fold increase in extracellular CKX activity was recorded in the ZmCKX5–GFP culture, CKX activity in the cell lysate of ZmCKX5–GFP was increased 77-fold compared to WT. In addition, the intracellular localization of ZmCKX5-GFP and ZmCKX8-GFP fusion proteins was also confirmed using western blot analysis (Supplementary Fig. 2.) Inasmuch as ZmCKX5 displays the second-highest specific activity after ZmCKX1, the value recorded in the culture media may be considered insignificant and attributed to lysis of some cells.

Cells expressing ZmCKX6–GFP, meanwhile, showed no protein expression overall but nevertheless survived the antibiotic

Fig. 2. Stable expression of ZmCKX-GFP fusion proteins in Arabidopsis suspension cells and maize tissue. (A) Schematic representation of CaMV35S:ZmCKX-GFP constructs used for stable transformation. Constructs carry CaMV35S promoter, maize ZmCKX, attB recombination sites, GFP and T*nos* terminator genes. Arabidopsis suspension cells were stably transformed with plasmid constructs CaMV35S:ZmCKX-GFP (B, C). Lines ZmCKX2ΔN_{term} and ZmCKX9ΔN_{term} lack the N-terminal signal sequences Met^1-His^{42} and Met^1-His^{43}, respectively. Free GFP constitutes a control plasmid carrying GFP under the control of CaMV35S promoter. Green channel (denoted as GFP, left column) of free GFP and ZmCKX–GFP constructs and transmission light images (DIC, middle column) were captured and then merged (right column). Rows designated with an asterisk contains image representing the initial phase, shortly after establishing the culture, whereas all other images show the situation in stabilized cell culture. Intact vacuoles were isolated from cultured cells expressing ZmCKX8-GFP fusion protein and visualized under a scanning confocal microscope (D). Arabidopsis cells expressing ZmCKX9-GFP fusion protein were stained with ER tracker™ Red dye (E). Green channel (denoted as GFP, left column) of ZmCKX9-GFP construct and ER tracker™ Red (ER tracker, middle column) were captured and then merged (right column). Co-localization of the two signals is represented by yellow color. ZmCKX2–YFP fusion protein was stably expressed in root cells of a maize plant 4 days old (F). Scale bars indicate 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


A
CaMV35S
ZmCKX
GFP
Tnos
attB1
attB2
B
GFP
DIC
merge
free GFP
ZmCKX2*
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ZmCKX2ΔN term *
ZmCKX4a
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ZmCKX9ΔN term *
ZmCKX10*
ZmCKX12
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GFP
ER tracker
merge
ZmCKX9
F
YFP

selection. Total CKX activity in the cell lysates and media of the ZmCKX6–GFP culture was comparable to that of the control cell cultures. As mentioned above, ZmCKX6 carries a mutation in the conserved HFG motif when compared to all functional CKX enzymes across the entire plant kingdom. Although this amino acid change is responsible for the loss of CKX activity, the precise role of this conserved motif is not known. It can be presumed that the mutation in the HFG motif is further accountable for the selective degradation of the protein and thus no functional protein can be detected *in planta* neither in the CKX activity assay nor in the western blot analysis (Supplementary Fig. 2.).

The measurements above together with our GFP-tagging experiments are supportive of the conclusion that the ZmCKX2, ZmCKX4a, ZmCKX5, ZmCKX8, and ZmCKX12 isozymes are targeted to the vacuole. To further prove this hypothesis, intact vacuoles were isolated from stably transformed *Arabidopsis Ler* suspension cells expressing ZmCKX8–GFP fusion protein. As shown in Fig. 2D, isolated vacuoles show a green fluorescent signal corresponding to ZmCKX8–GFP fusion protein.

In the case of the ZmCKX9 isozyme, the predictions of putative signal peptides were inconsistent and misleading. Transient expression of ZmCKX9–GFP in maize protoplasts confirmed association with the ER, similarly to that of other putatively apoplastic ZmCKX isozymes. The expression pattern of the ZmCKX9–GFP fusion protein in *Arabidopsis* suspension cells was suggestive of the ER, with its typical network-like structures (Fig. 2C). The signal pattern was consistent at all times during the selection and stabilization of this cell line. To further confirm localization of this isoform in the ER compartment, ZmCKX9–GFP cells were stained with ER tracker™ Red to visualize the ER compartment and then observed under a confocal microscope. As shown in Fig. 2E, the green signal of ZmCKX9–GFP co-localized with the red signal of the ER tracker™ Red and thus the experiment clearly confirmed the localization of ZmCKX9–GFP to the ER. Next, the mutant of ZmCKX9 was expressed in *Arabidopsis* suspension cells to prove the presence of ER-targeting signal sequence localized at the protein N-terminus. Similarly to ZmCKX2ΔN_{term}(1–42)-GFP cell line, ZmCKX9ΔN_{term}(1–43)-GFP mutant showed again cytosolic and nuclear distribution (Fig. 2C). When compared to the WT control, the specific CKX activity of the ZmCKX9 overexpressing line was increased by 29- and 6-fold in the cell lysate and in the culture media, respectively. These data confirmed that a substantial portion of ZmCKX9–GFP was localized intracellularly. This is in agreement with the result of the western blot analysis (Supplementary Fig. 2.). Nevertheless, due to the significantly increased CKX activity in the culture media, partial secretion of ZmCKX9–GFP to the apoplast cannot be excluded. On the other hand, the retention of the protein at the ER is undoubtedly more intensive than in the case of apoplastic ZmCKX1.

3.5. Cytokinin depletion in ER- and vacuolar-localized CKX-overexpressing lines

Additionally, the effects of vacuolar- and ER-localized CKX overexpression on the CK status of cells and vacuoles were investigated. To this end, ZmCKX8 was selected as representative of the vacuolar CKX form and compared with the CK profile of the ER-localized ZmCKX9 and the WT control. The levels of dihydrozeatin (DHZ) derivatives in both vacuoles and the whole-cell extracts were below the limit of detection (Fig. 3). The most abundant CKs in the cells, as well as vacuoles, were isopentenyladenine (iP) and *cis*-zeatin (*c*Z) derivatives. The levels of *trans*-zeatin (*t*Z) derivatives, meanwhile, were very low in contrast to typical CK profiles measured in intact *Arabidopsis* plants (Werner et al., 2003). On the other hand, *c*Z derivative content was high even

A

	WT	ZmCKX8	ZmCKX9
Total *t*Z-CK	0.22	0.05	0.05
Total *c*Z-CK	25.05	13.93	7.97
Total DHZ-CK	ND	ND	ND
Total iP-CK	90.75	15.15	4.12

B

	WT	ZmCKX8	ZmCKX9
Total *t*Z-CK	6.41	2.06	2.03
Total *c*Z-CK	323.46	217.99	413.73
Total DHZ-CK	ND	ND	ND
Total iP-CK	986.47	49.88	74.53

Fig. 3. Cytokinin content in Arabidopsis suspension cells (A) and isolated intact vacuoles (B). The heat map shows the average accumulation of all CK-type derivatives. The value in each block indicates mean of two measurements. The concentration in A is expressed in pmol g^{-1} of fresh weight, whereas those in B are expressed in fmol per 1 million vacuoles. *t*Z – *trans*-zeatin, *c*Z – *cis*-zeatin, DHZ – dihydrozeatin, and iP – isopentenyladenine. ND – value below the limit of detection.

though it is usually very low in the vegetative tissues of intact plants (Gajdošová et al., 2011). Therefore, the ratio of *t*Z to *c*Z indicates that CK homeostasis in *Arabidopsis Ler* suspension cell cultures will be far different from that of intact plants.

Constitutive ZmCKX overexpression resulted in a strong depletion of all CK forms within the cell. Even though ER-localized ZmCKX9 is the least active enzyme of the entire ZmCKX family (Table 2), its overexpression in suspension cells had a stronger effect on CK homeostasis than did that of ZmCKX8 (Fig. 3A). As expected, the overexpression of vacuolar ZmCKX8 had a strong depletion effect on CK content in the isolated vacuoles (Fig. 3B). This negative effect was considerable, too, in the isolated vacuoles of the ZmCKX9 overexpressing line. Similar trends were observed in both the active as well as inactive CK species (data not shown). The decrease of vacuolar CK content in ZmCKX9–GFP vacuoles points to CK transport across the tonoplast membrane. This decrease is most probably due to the fact that the CK free bases are depleted in cytosol and/or ER by overproduced ZmCKX9–GFP prior to their transport to the vacuoles.

Expression of the ZmCKX10–GFP fusion protein in *Arabidopsis* cells showed an intensive cytosolic and nuclear targeting during the early phases of the stable line generation (Fig. 2C). Expression of the protein significantly decreased in the process of subsequent selection and stabilization of the culture, however, and finally resulted in non-elevated CKX activity in the stabilized culture. The identical performance was observed in three independent trials to derive the stable ZmCKX10–GFP culture. In the *Arabidopsis* plants, a CK deficiency caused by constitutive overexpression of the apoplastic or the vacuolar CKXs results in reduced stem growth and an enlarged root system (Werner et al., 2001). In contrast, the opposite effects were observed in the case of cytosolic AtCKX7-overexpressing plants (Köllmer et al., 2014). Here, the strong depletion in *c*Z and its derivatives caused a formation of only short, early terminating primary roots due to reduced apical meristem growth. These results are in sharp contrast to the situation of plants overexpressing other CKX genes, thus pointing to the importance of cytosolic CK homeostasis in the regulation of meristematic cell activity. Some parallels can be found between fast-cycling

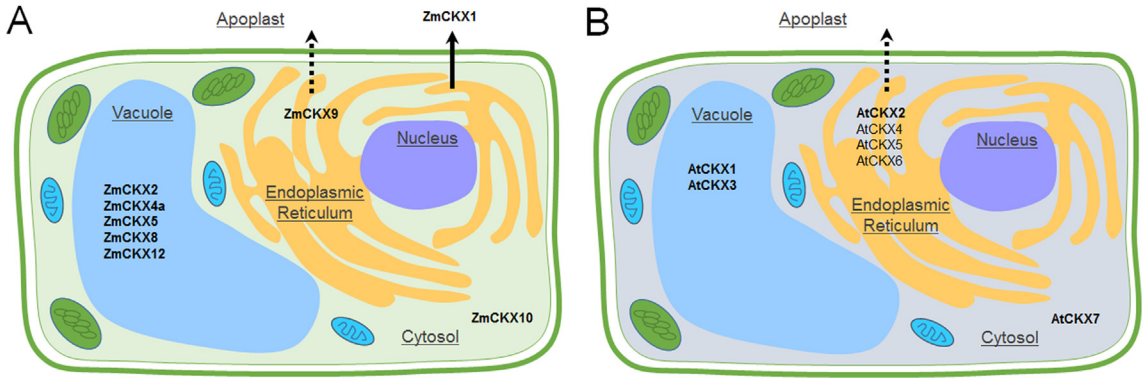

Fig. 4. Subcellular localization of CKX isozymes in *Zea mays* L. (A) and in *Arabidopsis thaliana* (B). Localization of isoforms shown in bold was proven experimentally while localization of those in non-bold are proposed only on the basis of bioinformatic data. Full arrows indicate experimentally proven secretion of CKX isozymes to the extracellular space; broken-line arrows indicate possible secretion based on bioinformatic analyses or the presented experiments. Small green and pink ovals represent chloroplast and mitochondria, respectively. Figure was prepared based upon our results and those of Werner et al (Werner et al., 2003); Galuszka et al (Galuszka et al., 2005); Šmehilová et al (Šmehilová et al., 2009). and Köllmer et al (Köllmer et al., 2014). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Arabidopsis suspension cells and root meristematic cells. We can thus hypothesize that the overproduction of cytosolic CKX isoform in suspension cells was eventually silenced by a random mutation caused by a strong negative selection pressure induced as a result of the CK homeostasis disturbance in the cytosol. This hypothesis is supported by the fact that western blot analysis has not confirmed the presence of ZmCKX10-GFP fusion protein (data not shown).

3.6. *Stable expression of ZmCKX2–YFP in maize root under the control of native promoter*

The use of constitutive promoter was favored over native promoters for successful signal detection of GFP–protein fusions because CKX enzymes are generally low-abundance proteins whose expression fluctuates immensely during ontogenesis (Galuszka et al., 2001; Vyroubalová et al., 2009; Podlešáková et al., 2012; Zalabák et al., 2014). Expression under the control of native promoters would probably provide no or a very weak signal. To increase the credibility of ZmCKX family localization using a heterologous expression system, finally one of the ZmCKXs fused with YFP under the control of their native promoters and other native regulatory sequences provided a satisfactory signal to follow its subcellular localization. The fluorescent signal of ZmCKX2–YFP was detected in the maize root cells of a stably transformed plant. Transgenic plants were prepared by integration of the cassette encompassing coding sequence of *ZmCKX2* (including introns) C-terminally fused with citrine YFP flanked with both 5′ and 3′ UTR regions. The expression pattern of the ZmCKX2–YFP fusion protein in maize root cells was very similar to that observed in *Arabidopsis* suspension cells (Fig. 2F). Taken together, our results clearly show that the majority of maize CKX isozymes are localized to the vacuoles. CK profiling of isolated intact vacuoles confirms that these isoforms are functional and actively control the vacuolar CK homeostasis. This conclusion is in agreement with the results of our recent study demonstrating that the level of active cytokinins within plant vacuoles is negligible (Jiskrová et al., 2016). Since the active cytokinin forms are the substrates of CKX isozymes, their level is therefore decreased via CKX activity in plant vacuoles. Our data also suggest that there must be some transport of CK from cytosol to the vacuoles across the tonoplast membrane.

Nevertheless, the physiological context of the apparent redundancy in the number of CKX isozymes catalyzing CK degradation in vacuoles remains unclear. It can be supposed that the vacuolar localization together with the specific tissue expression pattern, substrate preference, and rate of reaction plays an important role in fine-tuning the intracellular CK status.

4. Conclusions

The maize genome contains a small gene family encoding for CKX isozymes. Eleven out of 13 enzymes had been biochemically characterized in our previous studies (Šmehilová et al., 2009; Zalabák et al., 2014). In addition to their biochemical properties and distinct spatial and temporal expression pattern, maize CKX isoforms show diverse subcellular targeting. Until now, only two ZmCKX isozymes had been localized. While ZmCKX1 had been shown to be apoplastic, ZmCKX10 was localized to the cytosol and nucleus (Šmehilová et al., 2009). In this work, ZmCKX proteins were C-terminally tagged with GFP or YFP and transiently or stably expressed in maize protoplasts, *Arabidopsis* suspension culture, or intact maize plants. Except for ZmCKX10, all the other ZmCKX isozymes, including ZmCKX1, were shown to be transiently associated with the ER. In stably transformed cells, only ZmCKX9 was permanently associated with the ER. Nevertheless, its subsequent partial secretion to the apoplast cannot be excluded, as enhanced CKX activity was determined in the culture media similarly as in the case of ZmCKX1. Surprisingly, all the other ZmCKX isoforms, namely ZmCKX2, ZmCKX4a, ZmCKX5, ZmCKX8, and ZmCKX12, were proven to be localized in the vacuoles in this heterologous expression system. The correctness of the predicted vacuolar targeting of one of these CKX isozymes (ZmCKX2) was further confirmed by identical compartmentation in maize root cells when expression of the YFP-fused enzyme was driven by its native regulatory sequences.

Our study shows for the first time in a species other than the *Arabidopsis* model the general pattern of CK catabolism enzyme distribution on the subcellular level. Two out of 7 AtCKX isozymes are vacuolar, 1 is cytosolic, and 1 was proven to be ER-associated and subsequently most probably secreted out of the cell in *Arabidopsis* (Werner et al., 2001, 2003). There is strong bioinformatic evidence suggesting that the last three AtCKX enzymes are after their processing at the ER secreted out of the cell (Werner et al., 2003; Köllmer et al., 2014). As the *CKX* gene family has high synteny to other important cereal species with fully sequenced genomes (rice, barley (Mameaux et al., 2012);), the depicted maize model of CKX distribution can be generalized to all other cereals. It

is clear that CK homeostasis in monocots is regulated in the same compartments as in the *Arabidopsis* model, but more isozymes are bound to control CK levels in the vacuoles (Fig. 4). In contrast to *Arabidopsis*, only one isozyme, ZmCKX1, is predestined to regulate CK levels specifically in the apoplast and thus play a key role in maize CK metabolism.

Contributions

D.Z., O.P. designed and performed experiments, analyzed data and prepared the manuscript; P.J., E.J., K.Š. performed experiments; O.Š., D.J. and A.M contributed to localization analyses; O.N. performed UPLC-MS measurements; P.G. designed experiments, analyzed data, supervised the project and edited the manuscript.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.03.013.

References

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.

Bajguz, A., Piotrowska, A., 2009. Conjugates of auxin and cytokinin. Phytochemistry 70, 957–969.

Bannai, H., Tamada, Y., Maruyama, O., Nakai, K., Miyano, S., 2002. Extensive feature detection of N-terminal protein sorting signals. Bioinformatics 18, 298–305.

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.

Christensen, A.H., Sharrock, R.A., Quail, P.H., 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol. Biol. 18, 675–689.

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., 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.

Gajdošová, S., Spíchal, L., Kamínek, M., Hoyerová, K., Novák, O., Dobrev, P.I., Galuszka, P., Klíma, P., Gaudinová, A., Žižková, E., Hanuš, J., Dančák, M., Trávníček, B., Pešek, B., Krupička, M., Vaňková, R., Strnad, M., Motyka, V., 2011. Distribution, biological activities, metabolism, and the conceivable function of cis-zeatin-type cytokinins in plants. J. Exp. Bot. 62, 2827–2840.

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

Galuszka, P., Frébortová, J., Luhová, L., Bilyeu, K.D., English, J.T., Frébort, I., 2005. Tissue localization of cytokinin dehydrogenase in maize: possible involvement of quinone species generated from plant phenolics by other enzymatic systems in the catalytic reaction. Plant Cell Physiol. 46, 716–728.

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.

Gomord, V., Denmat, L.A., Fitchette-Lainé, A.C., Satiat-Jeunemaitre, B., Hawes, C., Faye, L., 1997. The C-terminal HDEL sequence is sufficient for retention of secretory proteins in the endoplasmic reticulum (ER) but promotes vacuolar targeting of proteins that escape the ER. Plant J. 11, 313–325.

He, Z.H., Cheeseman, I., He, D., Kohorn, B.D., 1999. A cluster of five cell wall-associated receptor kinase genes, Wak1–5, are expressed in specific organs of Arabidopsis. Plant Mol. Biol. 39, 1189–1196.

Hirose, N., Makita, N., Kojima, M., Kamada-Nobusada, T., Sakakibara, H., 2007. Overexpression of a type-A response regulators alters rice morphology and cytokinin metabolism. Plant Cell Physiol. 48, 523–539.

Jiskrová, E., Novák, O., Pospíšilová, H., Holubová, K., Karády, M., Galuszka, P., Robert, S., Frébort, I., 2016. Extra- and intracellular distribution of cytokinins in the leaves of monocots and dicots. N. Biotechnol. http://dx.doi.org/10.1016/j.nbt.2015.12.010.

Kakimoto, T., 2001. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. Plant Cell Physiol. 42, 677–685.

Köllmer, I., Novák, O., Strnad, M., Schmülling, T., Werner, T., 2014. Overexpression of the cytosolic cytokinin oxidase/dehydrogenase (CKX7) from Arabidopsis causes specific changes in root growth and xylem differentiation. Plant J. 78, 359–371.

Kowalska, M., Galuszka, P., Frébortová, J., Šebela, M., Béres, T., Hluska, T., Šmehilová, M., Bilyeu, K.D., Frébort, I., 2010. Vacuolar and cytosolic cytokinin dehydrogenases of *Arabidopsis thaliana*: heterologous expression, purification and properties. Phytochemistry 71, 1970–1978.

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.

Mameaux, S., Cockram, J., Thiel, T., Steuernagel, B., Stein, N., Taudien, S., Jack, P., Werner, P., Gray, J.C., Greenland, A.J., Powell, W., 2012. Molecular, phylogenetic and comparative genomic analysis of the cytokinin oxidase/dehydrogenase gene family in the *Poaceae*. Plant Biotechnol. J. 10, 67–82.

Mathur, J., Szabados, L., Schaefer, S., Grunenberg, B., Lossow, A., Jonas-Straube, E., Schell, J., Koncz, C., Koncz-Kálmán, Z., 1998. Gene identification with sequenced T-DNA tags generated by transformation of Arabidopsis cell suspension. Plant J. 13, 707–716.

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowská, D., Tabata, S., Sandberg, G., Kakimoto, T., 2006. Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc. Nat. Acad. Sci. U. S. A. 103, 16598–16603.

Mohanty, A., Luo, A., DeBlasio, S., Ling, X., Yang, Y., Tuthill, D.E., Williams, K.E., Hill, D., Zadrozny, T., Chan, A., Sylvester, A.W., Jackson, D., 2009. Advancing cell biology and functional genomics in maize using fluorescent protein-tagged lines. Plant Physiol. 149, 601–605.

Mohanty, A., Yang, Y., Luo, A., Sylvester, A.W., Jackson, D., 2009. Methods for generation and analysis of fluorescent protein-tagged maize lines. In: Scott, M.P. (Ed.), Methods in Molecular Biology: Transgenic Maize. Humana Press, USA, pp. 71–89.

Mrízová, K., Jiskrová, E., Vyroubalová, Š., Novák, O., Ohnoutková, L., Pospíšilová, H., Frébort, I., Harwood, W., Galuszka, P., 2013. Overexpression of cytokinin dehydrogenase genes in barley (Hordeum vulgare cv. Golden Promise) fundamentally affects morphology and fertility. PLoS One 8, e79029.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., Kimura, T., 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J. Biosci. Bioeng. 104, 34–41.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

Podlešáková, K., Zalabák, D., Čudejková, M., Plíhal, O., Szüčová, L., Doležal, K., Spíchal, L., Strnad, M., Galuszka, P., 2012. Novel cytokinin derivatives do not show negative effects on root growth and proliferation in submicromolar range. PLoS One 7, e39293.

Robert, S., Zouhar, J., Carter, C., Raikhel, N., 2007. Isolation of intact vacuoles from Arabidopsis rosette leaf–derived protoplasts. Nat. Protoc. 2, 259–262.

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.

Takei, K., Sakakibara, H., Sugiyama, T., 2001. Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. J. Biol. Chem. 276, 26405–26410.

Takei, K., Yamaya, T., Sakakibara, H., 2004. Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-zeatin. J. Biol. Chem. 279, 41866–41872.

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.

Zalabák, D., Galuszka, P., Mrízová, K., Podlešáková, K., Gu, R., Frébortová, J., 2014. Biochemical characterization of the maize cytokinin dehydrogenase family and cytokinin profiling in developing maize plantlets in relation to the expression of cytokinin dehydrogenase genes. Plant Phys. Biochem. 74, 283–293.