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RESEARCH PAPER

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

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Abstract

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

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

Introduction

Cytokinins are phytohormones that serve as important signalling molecules in plant cells. As hormones, they control cell division and influence apical dominance, leaf senescence, fruit development, and other processes (Mok and Mok, 2001). To find out how cytokinins carry out signalling functions, it is critical to understand the regulation of cytokinin homeostasis and activity in plant tissues. Cytokinin dehydrogenases (CKXs; EC 1.5.99.12) play an

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essential role in cytokinin regulation through irreversible degradation of the phytohormone. They inactivate the hormone molecule by cleavage of the isoprenoid side chain of *trans*-zeatin, N^6-(Δ^2-isopentenyl)adenine, and their ribosides to produce adenine or adenosine and the corresponding aldehydes (for a review, see Popelková *et al.*, 2004). A *CKX* gene was first cloned from maize by Morris *et al.* (1999) and simultaneously by Houba-Hérin *et al.* (1999). Shortly afterwards, it became apparent that plants contain small families of these genes. To date, such gene families have been identified and characterized in varying degrees in maize, *Arabidopsis thaliana*, rice, wheat, and barley (Bilyeu *et al.*, 2001, 2003; Galuszka *et al.*, 2004; Massonneau *et al.*, 2004; Popelková *et al.*, 2004). Sequence analysis of the first cloned *CKX* gene from maize (*ZmCKX1*, GenBank accession no. NM_001112121) revealed an N-terminal signal peptide that was predicted to target the enzyme to the cell secretory pathway (Morris *et al.*, 1999). Identification of glycosylation sites in the protein was also consistent with this prediction. Indeed, recombinant production of ZmCKX1 with its native signal peptide resulted in secretion from the yeast, *Pichia pastoris*, and from moss protoplasts (Houba-Hérin *et al.*, 1999; Morris *et al.*, 1999; Bilyeu *et al.*, 2001). The structure of the glycosylated protein has been determined recently (Malito *et al.*, 2004). CKX enzymes are oxidoreductases that can catalyse the degradation of cytokinins with molecular oxygen as an electron acceptor (oxidase reaction) or with other electron acceptors in a dehydrogenase reaction (Galuszka *et al.*, 2001; Laskey *et al.*, 2003; Frébortová *et al.*, 2004). For example, in studies of ZmCKX1, the potential of either reaction pathway was demonstrated (Frébortová *et al.*, 2004). Overexpression of CKX-encoding genes typically resulted in a cytokinin deficiency phenotype showing retarded stem growth and excessive root system development (Werner *et al.*, 2003; Galuszka *et al.*, 2004).

In further studies using immunohistochemical methods to detect native ZmCKX1 in maize tissues, this enzyme was localized to the apoplast region (Galuszka *et al.*, 2005). The extensive characterization of the membrane association of ZmCKX1 was prevented due to relatively low expression in maize tissues and lack of an antibody specific to ZmCKX1 (not cross-reacting with other CKX isozymes). In all plants with sufficient sequence data for analysis, targeted forms of *CKX* occur as multiple gene copies. Another maize CKX enzyme (CKO3) was secreted to the culture medium when expressed with its native signal sequence in a heterologous *Yarrowia lipolytica* expression system (Massonneau *et al.*, 2004). The fluorescence signals of two *Arabidopsis* CKX proteins fused with the green fluorescent protein (GFP) tag were detected in vacuoles in root cells as well as in leaf cells (Werner *et al.*, 2003). Non-targeted variants of *CKX* genes (lacking predicted N-terminal signal peptide sequences) have also been identified *in silico* in several plant species (*Oryza sativa*, *Glycine max*, *Hordeum vulgare*, and *Triticum aestivum*; unpublished results). In *A. thaliana*, a single copy of the only non-targeted CKX, AtCKX7, was functionally characterized (Galuszka *et al.*, 2007).

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

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

Materials and methods

Isolation of the ZmCKX10 *gene*

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

Site-directed mutagenesis of ZmCKX10

The open reading frame (ORF) for *ZmCKX10* was repaired in two positions using the QuikChange Site-Directed Mutagenesis Kit following the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Primers were designed according to the QuikChange® Primer Design Program (Stratagene). For the ATC to GTC change at position 376 (isoleucine to valine) the following primers were used: forward a376g_f 5′-GCTTCGCCGACGTCCCCGGCGGC-3′ and reverse a376g_r 5′-GCCGCCGGGGACGTCGGCGAAGC-3′. For the AAG to CAG change at position 688 (lysine to glutamine), a688c_f 5′-ACAGGGCGCCCCAGGCGGTGCGG-3′ and

a688c_r 5′-CCGCACCGCCTGGGGCGCCCTGT-3′ were used, respectively. As a template, 50 ng of vector DNA was used per reaction. The *ZmCKX10* ORF was repaired in two separate reactions each in 12 cycles with annealing at 68 °C. Mutated DNAs were analysed by sequencing.

Quantitative PCR (qPCR) analysis

Total RNA for reverse transcription was isolated using an RNAqueous® kit and Plant RNA Isolation Aid solutions (Applied Biosystems/Ambion, Austin, TX, USA). Isolated RNA was treated using a TURBO DNase-free™ kit (Ambion) to minimize the bias in PCR data caused by traces of genomic DNA contamination. First-strand cDNA was synthesized by RevertAid™ H Minus M-MuLV reverse transcription and random hexamer primers (Fermentas). Diluted cDNA samples were used as templates in real-time PCRs containing POWER SYBR® Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) and 300–900 nM of forward and reverse primers. The primers were designed using Primer Express 3.0 software (Applied Biosystems). RNA from each tissue was transcribed in two independent reactions and each cDNA sample was run in at least two technical replicates on a StepOnePlus™ Real-Time PCR System in a default program (Applied Biosystems). The specificity of the amplification was verified by melting curve analysis. The 18S small subunit rRNA gene (GenBank accession no. AF168884) was used as the endogenous control to normalize the variations in quality of RNA and efficiency of transcription among the samples. The sequences of the applied primers were designed as follow: 18S_f 5′-CCATCCCTCCGTAGTTAGCTTCT-3′ and 18S_r 5′-CCTGTCGGCCAAGGCTATATAC-3′ for 18S RNA, Zm1_f 5′-TGCAGGGCACCGACATC-3′ and Zm1_r 5′-CGTCCCACATGGATTTGTTG-3′ for *ZmCKX1* (reverse primer overlaps the exon2/3 junction), and Zm10_f 5′-CGACATCGCCGACTTCGA-3′ and Zm10_r 5′-GGGTCCCACTTGCTCTTGAG-3′ for *ZmCKX10* (reverse primer overlaps the exon 3/4 junction). For both genes, plasmid DNA containing the gene was used as a template to generate a calibration curve to determine the efficiency of PCRs. The efficiency of amplification was calculated from standard curves done for 10^2–10^9 gene copy numbers as 99.2% (R^2=0.998) for *ZmCKX1* and 101.2% (R^2=0.996) for *ZmCKX10*, respectively. The copy number was calculated for 1 ng of mRNA sample isolated from embryo, which was used as a calibrator for ΔΔCt relative quantification methods corrected by an efficiency factor.

Production and purification of recombinant ZmCKX10

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

pPICZA:*ZmCKX10* transformants were obtained using transformation of *P. pastoris* with linearized pPICZA:*ZmCKX10* vector by electroporation following the manufacturer's protocol (Invitrogen). The transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing 100 mg l^{-1} zeocin (Duchefa Biochemie BV, Haarlem, The Netherlands). Selected transformants were analysed for plasmid integration using PCR according to the manufacturer's protocol (Invitrogen). Selected clones were grown overnight in 50 ml of BMGY medium consisting of 1% yeast extract, 2% peptone, 1.34% Yeast nitrogen base without amino acids (YNB, Difco Laboratories, Detroit, MI, USA), 4×10^{-5}% D-biotin, 1% glycerol, buffered to pH 6.5 by K^+ phosphate buffer at 30 °C with extensive shaking at 230 rpm. The overnight culture was centrifuged at 1500 *g* for 5 min, washed, and resuspended to OD_{600}=1 in BMMY medium [1% yeast extract, 2% peptone, 1.34% YNB (Difco), 4×10^{-5}% D-biotin] buffered with K^+ phosphate buffer pH 6.5. The expression was induced by addition of methanol to a final concentration of 0.5%, and this concentration was maintained for 3 d. After 3 d, the cells were harvested by centrifugation at 1500 *g* for 5 min at 4 °C. The cells were broken using 0.45 mm glass beads (Sigma-Aldrich, St Louis, MO, USA) in a breaking buffer (50 mM sodium phosphate pH 7.4, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM EDTA, 5% glycerol). The lysate was concentrated using ultrafiltration on a Microcon YM-10 membrane (cut-off 10 kDa, Millipore, Billerica, MA, USA) and kept at 4 °C in 0.05 M TRIS-HCl pH 8.0. The concentrated proteins were loaded on an octyl-Sepharose CL-4B hydrophobic column (GE-Healthcare, Freiburg, Germany) connected to a BioLogic LP liquid chromatograph system (Bio-Rad, Hercules, CA, USA). After applying the sample, the column was washed with a descending step gradient of ammonium sulphate, and the eluate was fractionated. The fractions showing enzyme activity were pooled and concentrated using a stirred ultrafiltration cell (Millipore, Bedford, MA, USA) equipped with the YM-10 membrane. The purified ZmCKX10 was analysed using a Microflex MALDI-TOF LR20 mass spectrometer equipped with a nitrogen laser (Bruker Daltonic, Bremen, Germany) following a published protocol (Pospíšilová *et al.*, 2008).

CKX activity assay

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

The enzymatic reaction was stopped after incubation by adding 0.3 ml of 40% trichloroacetic acid (TCA), and the sample was centrifuged at 19 500 *g* for 5 min to remove

protein precipitate. After that, 0.2 ml of 4-aminophenol (2% solution in 6% TCA) was added to the supernatant followed by immediate (within 3 min) scanning of the absorption spectrum from 300 nm to 700 nm to determine the concentration of produced Schiff base with ε_{352}=15.2 mM^{-1} cm^{-1} (Frébort *et al.*, 2002).

For determination of the electron acceptor preference of ZmCKX1 (enzyme as described: Bilyeu *et al.*, 2001) and ZmCKX10 (produced as described above), the enzymatic activity was assayed with the following electron acceptors: 100 μM DCPIP, 250 μM Q_0, 250 μM ferricyanide (Lachema, Brno, Czech Republic), or without the acceptor (in the presence of oxygen). For the assessment of substrate specificity, a modified continual method for CKX activity measurement (Laskey *et al.*, 2003) was used with 100 μM DCPIP and a variety of substrates (50 μM): iP and isopentenyladenine 9-glucoside (iP9G) (both from Sigma-Aldrich) and isopentenyladenosine (iPR), isopentenyladenine 7-glucoside (iP7G), isopentenyladenosine 5-monophosphate (iPMP), *trans*-zeatin (tZ), *trans*-zeatin riboside (tZR), *cis*-zeatin (cZ), *cis*-zeatin riboside (cZR), *trans*-zeatin glucoside (tZ9G), and dihydrozeatin (dhZ), isopentenyladenine 7-glucoside (iP7G) (all from OlChemIm, Olomouc, Czech Republic). All substrates were dissolved in DMSO with a final concentration of 5% in the reaction mixture.

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

Generation of GFP translational fusions

A Modular Binary Construct System (Christopher Taylor Lab, Donald Danforth Plant Science Center, St Louis, MO, USA) was used for *GFP* gene fusions, binary vector construction, and subsequent transformation and overexpression of the *GFP*-tagged *ZmCKX1* and *ZmCKX10* genes in tomato hairy roots under a super ubiquitin (SU) promoter. The *ZmCKX10* ORF in pDrive was PCR amplified using the following primers: nsBam_f 5′-CG**GGATCC***ATGATGCTCGCGTACA*-3′, containing a *Bam*HI restriction site compatible with the *Bam*HI site in the *GFP* prepro region of the AKK1436 vector (bold), and nsBam_r 5′-CG**GGATCC***A*<u>*C*</u>*ACGGCGACG*-3′ containing a *Bam*HI restriction site (bold) and the 3′-untranslated *ZmCKX10* cDNA (italics) with a modification in the stop codon region (underlined). By omitting the CT of the stop codon of *ZmCKX10* and subsequent *Bam*HI restriction of the PCR product and AKK1436, an in-frame *ZmCKX10–GFP* fusion was produced. Similarly, an in-frame fusion of *ZmCKX1–GFP* was produced as follows. The *ZmCKX1* (GenBank accession no. NM_001112121) ORF present in the binary vector pROM30 (KD Bilyeu, unpublished) was PCR amplified using the primers: ZmBgl_f 5′- GA**AGATC**-**T***ATGGCGGTGGTTTATTACCTGC*-3′ containing a *Bgl*II site (bold), and ZmBgl_r 5′-GA**AGATCT**C<u>*G*</u>*TTGAAGATGTCCTGGCCG*-3′. By digesting the amplified product with *Bgl*II, a GATC overhang was created and subsequently ligated with the same overhang of *Bam*HI-digested AKK1436. The same PCR conditions were used as described in 'Isolation of the *ZmCKX10* gene'. To incorporate the GFP fusions into the new binary vector, a cloning cassette of AKK1436 containing the appropriate GFP-fused gene was cut out by *Pac*I digestion and ligated into *Pac*I-digested binary vector AKK1472B. A positive control for overexpression of just GFP was generated by ligation of the AKK1436 cloning cassette directly into the AKK1472B binary vector. The genes were fused with GFP after a short linker sequence between the genes and GFP (for details see Supplementary Fig. S1 available at *JXB* online).

Tomato hairy root transformation and cultivation conditions

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

Tomato seedlings (*Solanum lycopersicum* L., Peto 343) were grown in a growth chamber at 24 °C, with a 12 h photoperiod (illuminance 1000–1500 lux), for 6–8 d on MS medium at pH 5.8 solidified with 0.8% agar (extra pure; Merck, Darmstadt, Germany). Explants were prepared by cutting cotyledons from the seedlings immediately after their appearance from the seed coat. The cotyledons were wounded using a scalpel blade and were maintained with the adaxial leaf surface upwards on D1 agar, which consists of MS agar supplied with 100 mM *trans*-zeatin (OlChemIm), for 2 d. On the second day, the explants were saturated with transformed *A. rhizogenes* 15834 and subsequently cultivated on Gamborg's B-5 medium (Sigma-Aldrich) solidified with 1% agar (extra pure, Merck) pH 5.7 for 10–20 d. The regenerated root system represented a composite of transformed and wild-type (WT) tissues. The status of individual regenerated roots was evaluated by assessing GFP fluorescence with a SMZ800 Stereoscopic Microscope (Nikon, Kawasaki, Japan). Individual transgenic roots expressing

GFP were excised and placed on 7.0 cm filter paper overlaid on Gamborg's B-5 medium agar pH 5.7 that was amended with 150 mg l^{-1} timentin (Duchefa) and 2 mg l^{-1} bialaphos (Wako Chemicals GmBH, Neuss, Germany). Cultures were wrapped with Medipor porous adhesive plaster with a hydrophobic protected surface (Mediplast, Jilemnice, Czech Republic) and incubated at 26 °C in the dark to induce root elongation and branching.

Subcellular localization of GFP-fused proteins

The roots were excised from their taproot, embedded in 4% low-melting agarose, and sectioned into slices (50–100 μm thick) using a VT 1000 vibratome (Leica Microsystems, Wetzlar, Germany). The root sections were mounted in 100 mM phosphate-buffered saline (PBS) pH 6.5 prior to observation. For confocal microscopy, a IX-81 FluoView 1000 laser scanning inverted microscope fitted with image processing LCS Imaris 5.0.3 software and a 488 nm argon laser was used (Olympus, Tokyo, Japan). GFP emission of scanned root sections was recorded using a 505–525 nm band-pass filter. Prior to the experiment, the proper intensity of lasers was adjusted using control WT tomato hairy roots (induced with WT *A. rhizogenes* 15834).

Protoplasts

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

Analysis of transgenic roots

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

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

Phenotype determination

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

Results

The sequence of the ZmCKX10 *gene*

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

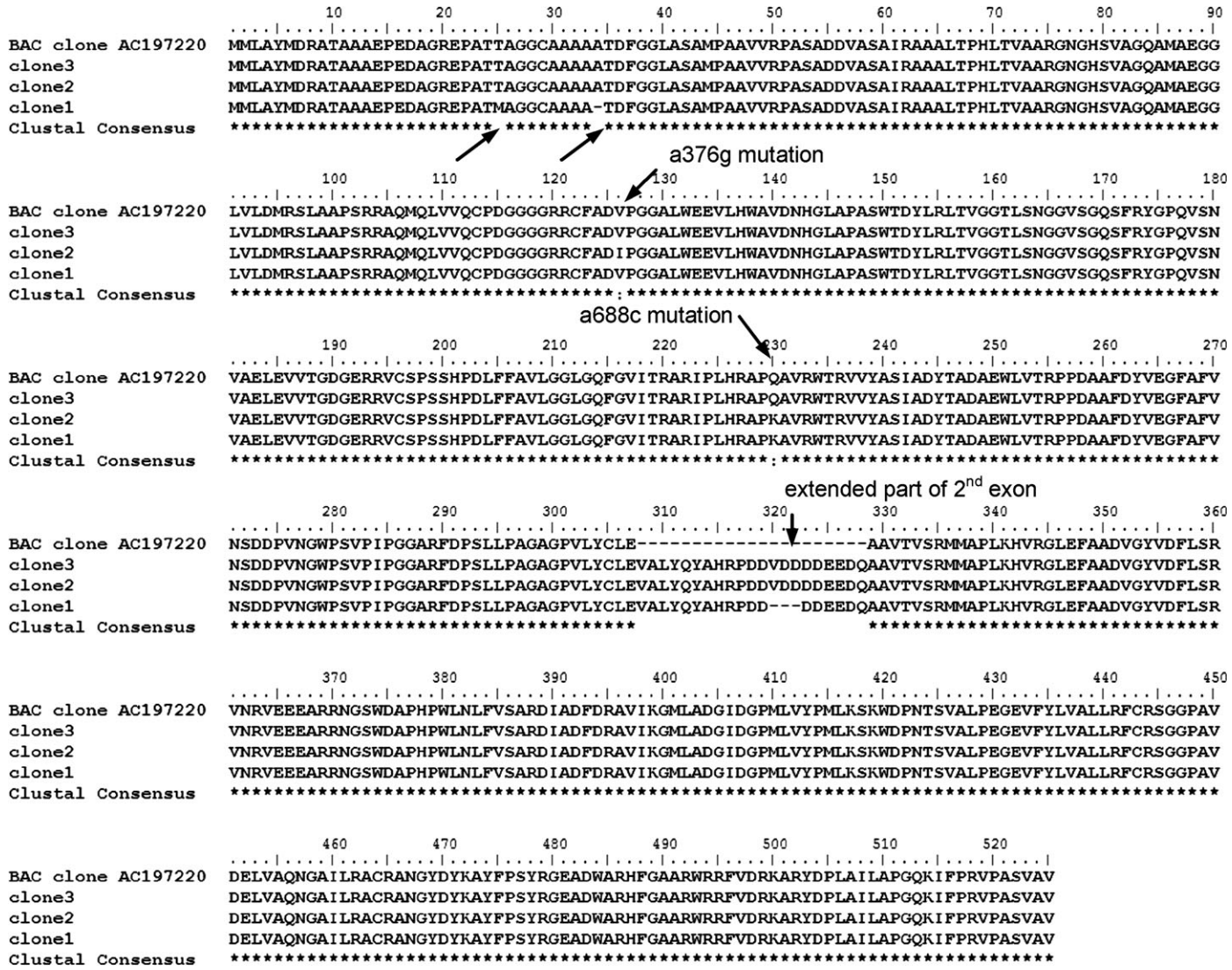

Fig. 1. ClustalW alignment of translated sequences of the predicted ZmCKX10 (part of the BAC clone AC197220.4) and the sequences of isolated ZmCKX10 clones. Identical and similar amino acids are marked with an asterisk and colon, respectively. Arrows show the positions where the sequences differ in comparison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

consisted of the full-length variant of the extended second exon and contained two single nucleotide variations, resulting in I126V and N230L mutations. The two mutations of clone 2 were repaired using site-directed mutagenesis to produce clone 3, which differs only in the presence of the extended second exon sequence in comparison with the original *ZmCKX10* sequence predicted from the database. Recombinant proteins of all three clones were prepared to investigate which sequence encoded a functional protein.

Organ-specific expression of two differentially targeted ZmCKX isoforms

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

Production of recombinant ZmCKX10

Three different versions of the *ZmCKX10* gene were expressed recombinantly in *P. pastoris.* Since the gene lacks a signal peptide, the expression was driven to the cytosol of the yeast host cell, where soluble ZmCKX10 was found.

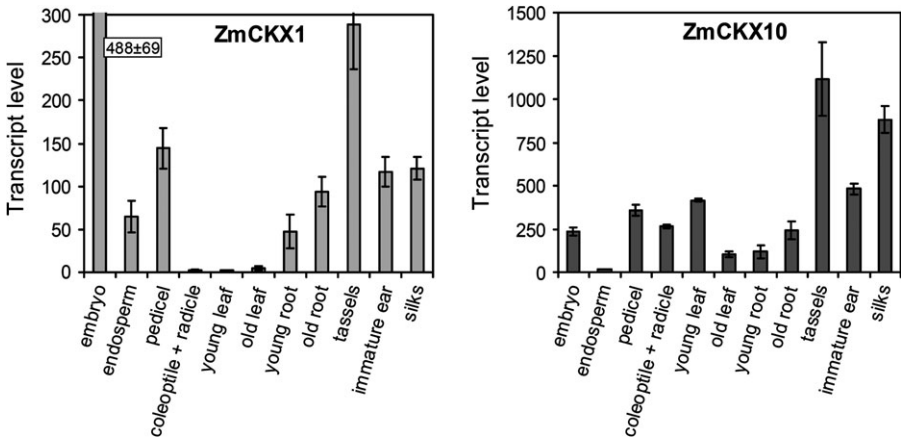

Fig. 2. Spatial and temporal expression profile of *ZmCKX1* and *ZmCKX10* genes. The transcript level determined by real-time RT PCR is given as the copy number of a particular gene per 1 ng of total RNA isolated from an embryo, which was used as a calibrator for ΔΔCt relative quantification methods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

The presence of functional ZmCKX10 protein was detected using a CKX activity assay (Frébort *et al.*, 2002; Galuszka *et al.*, 2007). All the three versions of the gene encoded active proteins, as demonstrated by CKX activity assay. Furthermore, there was no difference in the level of CKX activity among the three variants; the specific activity was ~8.5 pkat mg^{-1} of extracted intracellular proteins for all three clones when measured with iP as the substrate and DCPIP as the electron acceptor at pH 6.5, or iPR in the presence of Q_0 at pH 5.5. The recombinant protein encoded by clone 3 (GenBank accession no. FJ269181) was further purified by chromatography and characterized as a 56.3 kDa, 525 amino acid protein by mass spectrometry (see Supplementary Fig. S2 at *JXB* online).

Comparison of ZmCKX10 and ZmCKX1

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

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

Tomato hairy root transformation and subcellular localization of ZmCKX1 and ZmCKX10

Whereas ZmCKX1 was confirmed to be localized to the apoplast (Morris *et al.*, 1999; Galuszka *et al.*, 2005), ZmCKX10 was predicted to lack any translocation signal and therefore should function in the cytosol. To confirm the predicted localization of ZmCKX10 and compare it with ZmCKX1, the genes were fused on their C-termini with *GFP*. The fused genes were constitutively expressed in *A. rhizogenes*-induced tomato hairy roots. The transgenic root samples were analysed for fluorescence patterns using confocal microscopy. As shown in Fig. 4B, the diffuse fluorescence pattern attributable to transiently expressed ZmCKX10–GFP was similar to the fluorescence of transiently expressed GFP where a characteristic pattern can be found throughout the cytosol, accumulating along the plasma membrane and in nuclei (Grebenok *et al.*, 1997*a*, *b*; Köhler *et al.*, 1997; Haseloff and Siemering, 1998; Fig. 4A). It is known that GFP can enter the nuclei through nuclear pores, but large fusion proteins should not enter the nuclei. However, an experiment with a GFP dimer (55 kDa) and trimer (83 kDa) has shown that even though fluorescence remains primarily cytosolic, there are still remnants of fluorescence accumulating around the nuclei (von Arnim *et al.*, 1998). Accordingly, the 82 kDa ZmCKX10–GFP fusion protein containing no translocation signal showed a cytosolic location. Whereas ZmCKX10–GFP was localized in the cytosol, the ZmCKX1–GFP fluorescence pattern

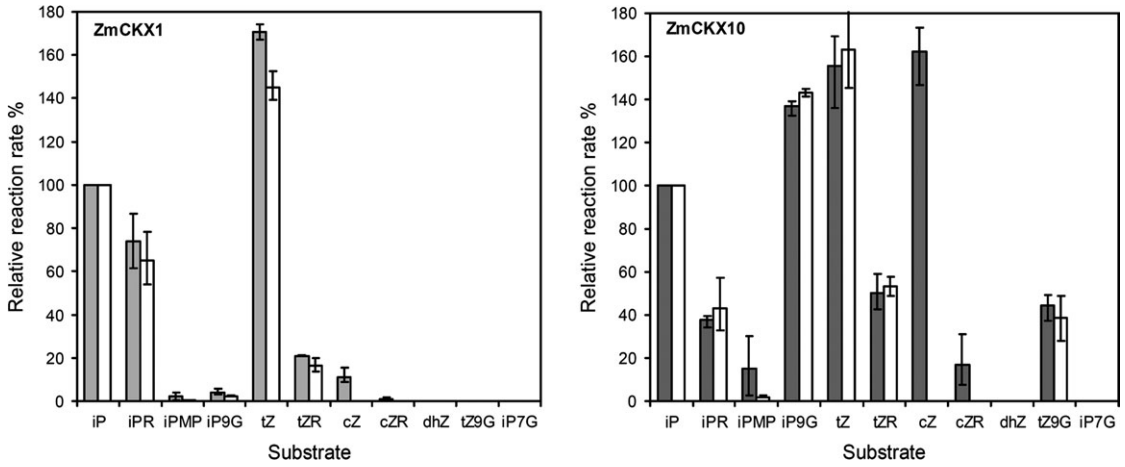

Fig. 3. Comparison of the substrate specificity of ZmCKX1 and ZmCKX10 expressed as relative reaction rates towards iP (specific activity measured with iP is given as 100%). The values were estimated by continuous (filled bars) and discontinuous (open bars) CKX activity assay. All analysed substrates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

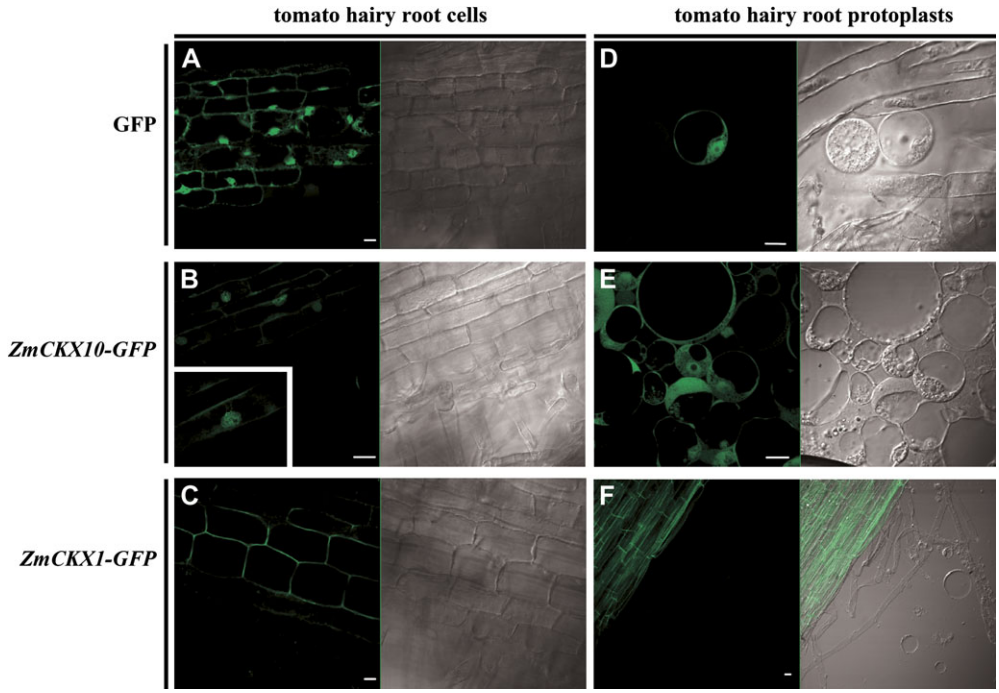

Fig. 4. Subcellular localization of GFP fusion proteins. Green channel and transmission light images were captured by confocal microscopy. (A) A control root, overexpressing SU:*GFP*, shows tthe ypical cytosolic localization of GFP with fluorescence along the plasma membrane in the cytosol and in the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

was visible only in the perimeters of the cells, confirming its localization in the apoplasts (Fig. 4C). To determine whether ZmCKX1 is actually associated with the plasma membrane, the cells were treated by cellulases to release protoplasts. Cellulase treatment led to the disappearance of the GFP fluorescence, indicating that there are no

interactions of ZmCKX1 with the plasma membrane (Fig. 4F). Protoplasts released from SU:*GFP* (Fig. 4D) and SU:*ZmCKX10–GFP* (Fig. 4E) were analysed as controls, and confirmed their intracellular fluorescence signals. The presence of the transgenes was confirmed using RT-PCR (see Supplementary Fig. S3 at *JXB* online).

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

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

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

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

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

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

Table 2. CKX activity assay of transgenic tomato hairy roots

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

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

* Measured with DCPIP as the electron acceptor and iP as the substrate at pH 6.5.
† Measured with Q_0 as the electron acceptor and iP9G as the substrate at pH 5.5.
‡ The values vary from 2373 pkat mg^{-1} to 8709 pkat mg^{-1} depending on the fluorescence intensity of the transgenic roots (probably due to varying efficiency of translation and correct folding).

Discussion

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

The gene coding for ZmCKX10 was successfully cloned as described above. According to obtained data, the cDNA of the *ZmCKX10* gene (GenBank accession no. FJ269181) consisted of 1578 bp. A discrepancy of 63 bp with the

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

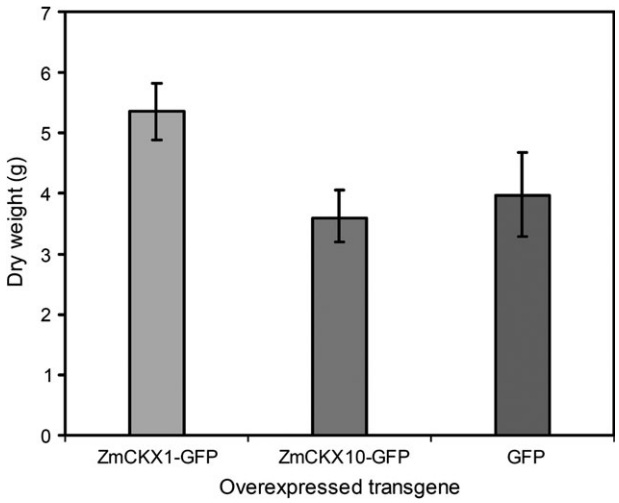

Fig. 5. Comparison of the rate of tissue production. The 21-d-old transgenic root tissue produced by the initial root was ground to a powder in liquid nitrogen, while the total weight of the tissue was measured. The values shown are means ±SD from three independent experiments with a minimum of eight root samples for each experiment. The roots overexpressing ZmCKX1–GFP correspond to 134.5% and ZmCKX10–GFP to 90.3%, when the dry weight of GFP-overexpressing roots is given as 100%.

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

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

CGGTGGACGCGCGTGGTGTACGCGAGCATCGCGGACTACACGGCGGACGCGGAGTGGCTGGTGACG
CGGCCCCCCGACGCGGCGTTCGACTACGTGGAGGGCTTCGCGTTCGTGAACAGCGACGACCCCGTG
AACGGCTGGCCGTCCGTGCCCATCCCCGGCGGCGCCCGCTTCGACCCGTCCCTCCTCCCCGCCGGC
GCCGGCCCCGTCCTCTACTGCCTGGAGGTGGCCCTGTACCAGTACGCGCACCGGCCCGACGACGTC
GACGACGACGATGAGGAGGACC**AGG**TAGGTAGCAGTAATTGCCAACCTCTCCCCCCGCTGAGACTT
GGCGCATTCCCGTACTTGACCCCCTCGCCCGCTCTGGCGTGTACTTTTCCGCGGGCAGGGCATGTC
TGACTCGCCTCGTCGTGTATCTCCCGCTGGATTCGGTGACGGGGGGGCTGCGTCCTGCCAAACCAA
ACCACCCTAGACTAGACAGACCCCCAGGGGCAGGGGTCGCGCCATTGGCCGCACGCGGGGACCGGC
GCCAGTGAGTGCGCCGCGCCGCACGGCCGCGCCCCGATCTCGCTCGCTCGCTCGCTGGTGATCGAA
TCGGCGCGTACAATGCGGCATGGCCCCGAGCCCCACACCCGCAGTGGCCGTGACGCGATTGCGCTG
CCTCCGGTCCGGCCCATGACCCAGCGGATCGCGTCGCGTCTTTTGGCAACGCCCGCGTCATCATAT
CGCGCTCTTTGTCGTCCCCACGGAGCACAGCGCAGCGCAGCGCAGCGCAGCCAACCTTTTCTCCGC
CACGCACGCTT**CGGCGGCATTCATTATTTGGATTTTGTTCCTACCGGTCGATCCGCGTCCGTCCGT**
GCACTGCAGGCGCTACCGTCATGCTGACCAACCCATTGCCATTGGTTTTGTTTCTTCTCTCTCTCT
CTCGCTCTCGTTGGTTATGGTTCGTGCGTGCCTGCAGGCGGCGGTGACCGTGAGCCGGATGATGGC
GCCGCTCAAGCACGTGCGGGGCCTGGAGTTCGCGGCGGACGTCGGGTACGTGGACTTCCTGTCCCG
CGTGAACCGGGTGGAGGAGGAGGCCCGGCGCAACGGCAGCTGGGACGCGCCGCACCCGTGGCTCAA
CCTCTTCGTCTCCGCGCGCGACATCGCCGACTTCGACCGCGCCGTCATCAAGGGCATGCTCGCCGA
CGGCATCGACGGGCCCATGCTCGTCTACCCTATGCTCAAGAGCAAGTG

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

Divergence of both isoenzymes was apparent in their preference for 9-glucosylated substrates. While ZmCKX10 degrades iP9G and tZ9G very effectively, the same substrates are almost unaffected by the activity of ZmCKX1. This is in agreement with the substrate specificity of *A. thaliana* cytosolic AtCKX7 and apoplastic CKXs demonstrated previously (Galuszka *et al.*, 2007). Turnover rates of both enzymes were enhanced by all the tested electron acceptors. Whereas the activity of apoplastic ZmCKX1 was enhanced by DCPIP the most (390-fold), none of the tested electron acceptors showed such a significant enhancement of the turnover rates for the cytosolic ZmCKX10 enzyme. However, it is notable that the activity of ZmCKX10 was 8.5-fold higher in the presence of ferricyanide and 7.5-fold higher in the presence of DCPIP than without any electron acceptor (in the presence of oxygen). Further biochemical studies concerning the catalytical mechanism should be performed to determine the ability of ZmCKX10 to use other electron acceptors or oxygen, and a possible mixed mode of action in the presence of artificial electron acceptors must be taken into account. Besides, the cytokinin depletion process in the cytosol might not need to be enhanced so significantly in this manner.

GFP fusions of the studied genes and their subsequent overexpression in tomato hairy roots resulted in the confirmation of the ZmCKX1 localization in the apoplast and established the predicted localization of ZmCKX10 to the cytosol. The images captured by confocal microscopy show that the fluorescent signal of ZmCKX10–GFP is localized to the cytosol and also in nuclei. However, the intensity of fluorescence of the ZmCKX10–GFP 82 kDa protein in nuclei is a remnant when compared with the fluorescent pattern of SU:*GFP* that was used as a positive control. The partial bidirectional diffusion of larger proteins back to the nuclei was described by von Arnim *et al.* (1998). Comparing the CKX activity of the roots overexpressing the GFP fusion proteins, an enormous difference was observed. As the CKX activity of the SU:*ZmCKX1–GFP* cell extract was 1564 times higher than that of the WT (and SU:*GFP*), no significant changes in the level of CKX activity of SU:*ZmCKX10–GFP* were detected when measured with DCPIP and iP as the substrate. When measured with Q_0 and iP9G as a preferred ZmCKX10 substrate, the CKX activity of the cell extract of SU:*ZmCKX10-GFP* was found to be 1.68 times higher than that of WT (and SU:*GFP*) roots and was also higher than in roots transformed with SU:*ZmCKX1–GFP*. SU:*ZmCKX1–GFP*-overexpressing roots were found to be no more active than the WT and SU:*GFP* under those assay conditions. The conditions for the measurement were chosen based on previous experience with determining CKX activity in cell extracts of plants overexpressing different *A. thaliana* CKX genes, where those cell extracts that were not so sensitive to iP as a substrate were much more sensitive to iP9G, especially from AtCKX7- (non-secreted) overexpressing plants (Galuszka *et al.*, 2007). Phenotypic characterization of the transgenic roots revealed a difference in the rate of root growth. The roots overexpressing SU:*ZmCKX1–GFP* were ascertained to form roots faster with 34.5% more tissue produced when compared with control roots. In contrast, roots overexpressing *ZmCKX10–GFP* showed an ~10% reduction in root formation. Hence, the root mass accumulation difference between SU:*ZmCKX10–GFP* and SU:*ZmCKX1–GFP* was ~44%. This difference indicates potentially opposing aspects of cytokinin regulation between the cytosol and apoplast impacting the physiology of root growth. Nevertheless, creating a whole plant overexpressing cytosolic CKX would provide more insight into the physiological implication of compartmentalization of cytokinin degradation. The results reported here suggest that non-targeted ZmCKX10 is a cytosolic enzyme of the maize CKX family that differs in enzymatic properties compared with targeted CKX isoforms. A considerable amount of further work is necessary to determine the nature of this unique cytosolic form of CKXs and how this class of enzyme (EC. 1.5.99.12) is contributing to the complexity of cytokinin homeostatic regulations in plants.

Supplementary data

Supplementary data are available at *JXB* online.

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References

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 KD, Cole JL, Laskey JG, Riekhof WR, Esparza TJ, Kramer MD, Morris RO. 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. *Plant Physiology* **125,** 378–386.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Collier R, Fuchs B, Walter N, Kevin Lutke W, Taylor CG. 2005. Ex vitro composite plants: an inexpensive, rapit method for root biology. *The Plant Journal* **43,** 449–457.

Emanuelsson O, Nielsen H, Brunak S, von Heijne G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300,** 1005–1016.

Frébort I, Šebela M, Galuszka P, Werner T, Schmülling T, Peč P. 2002. Cytokinin oxidase/cytokinin dehydrogenase assay: optimized procedures and applications. *Analytical Biochemistry* **306,** 1–7.

Frébortová J, Fraaije MW, Galuszka P, Šebela M, Peč P, Hrbáč J, Novák O, Bilyeu KD, English JT, Frébort I. 2004. Catalytic reaction of cytokinin dehydrogenase: preference for quinones as electron acceptors. *Biochemical Journal* **380,** 121–130.

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

Galuszka P, Frébortová J, Luhová L, Bilyeu KD, English JT, 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 and Cell Physiology* **46,** 716–728.

Galuszka P, Frébortová J, Werner T, Yamada M, Strnad M, Schmülling T, Frébort I. 2004. Cytokinin oxidase/dehydrogenase genes in barley and wheat: cloning and heterologous expression. *European Journal of Biochemistry* **271,** 3990–4002.

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. *Journal of Plant Growth Regulation* **26,** 255–267.

Grebenok RJ, Lambert GM, Galbraith DW. 1997*a*. Characterization of the targeted nuclear accumulation of GFP within the cells of transgenic plants. *The Plant Journal* **12,** 685–696.

Grebenok RJ, Pierson EA, Lambert GM, Gong F-C, Alfonso CL, Haldemann-Cahill R, Carrington JC, Galbraith DW. 1997*b*. Green-fluorescent protein fusions for efficient characterization of nuclear targeting. *The Plant Journal* **11,** 573–586.

Haseloff J, Siemering KR. 1998. The uses of green fluorescent protein in plants. In: Chalfie M, Kain S, eds. *Green fluorescent protein: strategies, applications and protocols*. New York: Wiley-Liss, Inc., 191–220.

Henke W, Herdel K, Jung K, Schnorr D, Loening SA. 1997. Betaine improves the PCR amplification of GC rich DNA sequences. *Nucleic Acids Research* **25,** 3957–3958.

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

Köhler RH, Zipfel WR, Webb WW, Hanson MR. 1997. The green fluorescent protein as a marker to visualize plant mitochondria *in vivo*. *The Plant Journal* **11,** 613–621.

Laskey JG, Patterson P, Bilyeu K, Morris RO. 2003. Rate enhancement of cytokinin oxidase/dehydrogenase using 2,6-dichlorindophenol as an electron acceptor. *Plant Growth Regulation* **40,** 189–196.

Malito E, Coda A, Bilyeu KD, Fraaije MW, Mattevi A. 2004. Structures of Michaelis and product complexes of plant cytokinin dehydrogenase: implications for flavoenzyme catalysis. *Journal of Molecular Biology* **341,** 1237–1249.

Massonneau A, Houba-Hérin N, Pethe C, Madzak C, Falque M, Mercy M, Kopečný D, Majira A, Rogowsky P, Laloue M. 2004. Maize cytokinin oxidase genes: differential expression and cloning of two new cDNAs. *Journal of Experimental Botany* **55,** 2549–2557.

Mok DWS, Mok MC. 2001. Cytokinin metabolism and action. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 89–118.

Morris RO, Bilyeu KD, Laskey JG, Cheikh NN. 1999. Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. *Biochemical and Biophysical Research Communications* **255,** 328–333.

Popelková H, Galuszka P, Frébortová J, Bilyeu KD, Frébort I. 2004. Cytokinin dehydrogenase: characterization and structure homology modelling of the flavoprotein catabolizing plant hormones cytokinins. In: Pandalai SG, ed. *Recent research developments in proteins*, Vol. 2. Kerala, India: Transworld Research Network, 63–81.

Pospíšilová H, Šebela M, Novák O, Frébort I. 2008. Hydrolytic cleavage of *N*6-substituted adenine derivatives by eukaryotic adenine and adenosine deaminases. *Bioscience Reports* **28,** 335–347.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673–4680.

Veach YK, Martin RC, Mok DWS, Malbeck J, Vaňková R, Mok MC. 2003. O-Glucosylation of *cis*-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. *Plant Physiology* **131,** 1374–1380.

von Arnim AG, Deng X-W, Stacey MG. 1998. Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. *Gene* **221,** 35–43.

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T. 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *The Plant Cell* **15,** 2532–2550.

Werner T, Motyka V, Strnad M, Schmülling T. 2001. Regulation of plant growth by cytokinin. *Proceedings of the National Academy of Sciences, USA* **98,** 10487–10492.