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Functional expression and purification of cytokinin dehydrogenase from *Arabidopsis thaliana* (AtCKX2) in *Saccharomyces cerevisiae*

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Abstract

Cytokinin dehydrogenase (CKX) is responsible for regulating the endogenous cytokinin content by oxidative removal of the side chain and seven distinct genes, *AtCKX1* to *AtCKX7*, code for the enzyme in *Arabidopsis thaliana*. The recombinant enzyme AtCKX2 was produced in *Saccharomyces cerevisiae* after expressing the corresponding gene from a plasmid (pDR197) or following chromosomal integration, under either the constitutive promoter *PMA1* or the inducible promoter *GAL1*. The recombinant protein was purified from yeast culture media using a sequence of chromatographic steps. The purified enzyme had a molecular mass of 61 kDa and a typical flavoprotein spectrum. The specific activity of the enzyme was 87.8 μkat g^{-1}, with isopentenyladenine as a substrate and 2,3-dimethoxy-5-methyl-*p*-benzoquinone as an electron acceptor. The pH optimum lay between 7.0 and 8.0, depending on the electron acceptor used. AtCKX2 reacts both with isoprenoid and aromatic cytokinins, the activity with isoprenoid cytokinins being two to three orders of magnitude higher. AtCKX2 prefers *p*-quinones and the synthetic dye 2,6-dichlorophenol indophenol as electron acceptors, although low reactivity with oxygen can also be observed. This study presents the first purification and characterization of the enzyme from *Arabidopsis thaliana*.

Additional key words: cytokinin metabolism, cytokinin oxidase.

Introduction

In plants, cytokinins are metabolized in a number of pathways that lead to compounds with modulated physiological activity (Mok *et al*. 2001). A complete irreversible inactivation is achieved through the action of cytokinin dehydrogenase (CKX; EC 1.5.99.12), a flavoprotein that oxidatively cleaves the N^6-side chain of cytokinins to form adenine and a side chain-derived aldehyde (Brownlee *et al.* 1975). The activity of CKX was first described in 1971 in a cultured tobacco tissue (Pačes *et al*. 1971). Since then, CKX has been identified and purified from many different plant species (Galuszka *et al*. 2000). CKX-like activity has also been identified in few lower organisms, such as the moss *Funaria hygrometrica* (Gerhäuser and Bopp 1990), the slime mold *Dictyostelium discoideum* (Armstrong and Firtel 1989) and the yeast *Saccharomyces cerevisiae* (Van Kast and Laten 1987). The presence of endogenous CKX in *D. discoideum* and *S. cerevisiae* is however dubious,

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Abbreviations: AtCKX2 - cytokinin dehydrogenase from *Arabidopsis thaliana* encoded by the gene *AtCKX2* (TAIR accession No. At2g19500); CKX - cytokinin dehydrogenase (EC 1.5.99.12); DCPIP - 2,6-dichlorophenol indophenol; DHZ - dihydrozeatin; iP - N^6-(2-isopentenyl)adenine; iP9G - N^6-(2-isopentenyl)adenine 9-glucoside; iPR - N^6-(2-isopentenyl)adenine 9-riboside; iPRMP - N^6-(2-isopentenyl)adenine 9-riboside-5'-monophosphate; MiP - N^6-methyl-isopentenyladenine; *m*-T - *m*-topolin; NBT - nitroblue tetrazolium chloride; *o*-T - *o*-topolin; *p*-T - *p*-topolin; Q_0 - 2,3-dimethoxy-5-methyl-1,4-benzoquinone; *t*-Z - *trans*-zeatin; *t*-ZR - *trans*-zeatin 9-riboside; Z9G - *trans*-zeatin 9-glucoside; ZmCKX1 - cytokinin dehydrogenase from *Zea mays* encoded by the gene *ZmCKX1* (GenBank accession no. AF044603).
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as homologous gene sequences were not found in these organisms (Schmülling *et al*. 2003).

The content of CKX is generally higher in roots than in shoots (Jones and Schreiber 1997); the best source of CKX being developing seeds (Jones *et al*. 1992, Galuszka *et al*. 2001). Isolation of the pure protein is, however, made difficult by its extremely low concentration in plant tissues. The cloning of a gene encoding CKX from maize (ZmCKX1) (Houba-Herin *et al*. 1999, Morris *et al*. 1999) presented new possibilities for the preparation of large quantities of the recombinant protein. *ZmCKX1* gene was cloned into yeasts *Pichia pastoris* and *Yarrowia lipolytica* and the protein was purified from cell-free culture media (Morris *et al.* 1999, Bilyeu *et al*. 2001, Kopečný *et al*. 2005). The availability of high amount of homogenous enzyme allowed detailed characterizations of enzyme properties, description of the catalytic mechanism and the crystallization of the protein (Bilyeu *et al*. 2001, Frébortová *et al.* 2004, Malito *et al*. 2004, Kopečný *et al*. 2005, Popelková *et al*. 2006). Cloning of *CKX* genes from *Arabidopsis thaliana* (Werner *et al*. 2001, 2003), *Dendrobium* orchid (Zang *et al*. 2003), *Hordeum vulgare* (Galuszka *et al*. 2004), and *Zea mays* (Massonneau *et al*. 2004) followed on the basis of gene sequence similarities.

Plants contain small *CKX* gene families (Popelková *et al*. 2004). In *A. thaliana*, seven distinct genes coding for CKX (*AtCKX1*-*AtCKX*7) were identified (Schmülling *et al*. 2003). Amino acid identity among AtCKX proteins is between 34 and 66 % (Schmülling *et al*. 2003). Individual *AtCKX* genes are expressed at different developmental stages and in different plant tissues (Werner *et al*. 2003). Although the role of CKX genes and proteins in plant development was extensively studied (Werner *et al*. 2001, 2003), individual CKX proteins from *A. thaliana* have not been purified and characterized to date.

One of the seven genes encoding CKX from *A. thaliana*, *AtCKX2*, has been previously shown to give the active protein in the yeasts *S. cerevisiae* and *P. pastoris* (Werner *et al*. 2001, Bilyeu *et al.* 2001). The protein was excreted into the culture media, in agreement with its predicted extracellular localization (Schmülling *et al*. 2003). In this work we report preparation of the yeast strain for production of recombinant AtCKX2 protein and its molecular characterization.

Materials and methods

Materials: The cDNA of *AtCKX2* was reported previously (Werner *et al*. 2001). The pDR197 plasmid that was prepared from pDR195 (Rentsch *et al*. 1995) introducing a sequence of *Sma*I-*Pst*I-*Eco*RI-*Eco*RV-*Hin*dIII-*Sal*I-*Xho*I-*Acc*65I-*Kpn*I restriction sites (TCGAGATCCC CCGGGCTGCA GGAATTCGAT ATCAAGCTTA TCGATACCGT CGACCTCGAG GGGGGGCCCG GTACCCAATT CGCCCTATAG TGAGTCGTAT TACGCGCG) into the multicloning site of pDR195 between *Xho*I and *Bam*HI (eliminating *Not*I-*Sac*II sites), was obtained from Dr. D. Rentsch (University of Bern, Switzerland). The yeast integration vector YIplac211 (Gietz and Sugino 1988), Genbank X75462, was donated by Dr. S. Lalonde (University of Tübingen, Germany). The expression plasmid pYES2::AtCKX2 was prepared as reported in Werner *et al.* (2001). *Escherichia coli* DH10B electrocompetent cells and T4 DNA ligase were from *Invitrogen* (Groningen, The Netherlands). The *Saccharomyces cerevisiae* strain used was 23344c (MATα *ura3*) which is derived from the wild type strain S1278b (Bechet *et al*. 1970).

Yeast nitrogen base (w/o aminoacids and ammonium sulfate) was from *Difco* (Detroit, MI, USA). YPAD medium was prepared from the commercial YPD medium (*Clontech*, Palo Alto, CA, USA) by adding 0.01 % adenine. All restriction enzymes were from *Fermentas* (St. Leon-Rot, Germany). For plasmid purification, Qiagen Mini and Midi kits were used. Galactose, nitroblue tetrazolium chloride (NBT) and vitamin K_1 were from *Fluka* (Buchs, Switzerland), vitamin K_3 and duroquinone from *Aldrich* (Millwaukee, WI, USA) and 1,4-naphthoquinone was from *BDH Chemicals* (Poole, England). 2,6-Dichlorophenol indophenol (DCPIP) was from *LOBA Feinchemie* (Fischamend, Austria). N^6-(2-isopentenyl)adenine 9-glucoside (iP9G), N^6-(2-isopentenyl)adenine 9-riboside-5'-monophosphate (iPRMP), *trans*-zeatin (Z), *trans*-zeatin 9-riboside (ZR), *trans*-zeatin 9-glucoside (Z9G), dihydrozeatin (DHZ), *o*-topolin (oT), *m*-topolin (mT), and *p*-topolin (pT), were from *OlChemIm* (Olomouc, Czech Republic). N^6-methyl-isopentenyladenine (MiP) that had been synthesized according to Wang and Letham (1995) was donated by Dr. K. Bilyeu (USDA, University of Missouri, Columbia, MO, USA). All other chemicals were from *Sigma* (St. Louis, MO, USA).

Plasmid-based expression under control of the constitutive promoter PMA1 (pDR197::AtCKX2): The plasmid *pCR Blunt II TOPO* (*Invitrogen*) with the *AtCKX2* insert in the antisense direction that had previously been used for the gene amplification and sequencing (Werner *et al*. 2001) was used as the starting material. The *AtCKX2* gene was cut out with *Bam*HI and *Pst*I and ligated into the multicloning site of pDR197 *E. coli*-yeast shuttle vector (containing an ampicillin resistance gene) that was opened with the same restriction endonucleases (Fig. 1*A*). The pDR197 vector contains a constitutive promoter *PMA1* and a transcription terminator *ADH* that allow functional expression of the protein in yeast. Yeast transformation with the expression plasmid was done by electroporation at 1.5 kV using a published protocol (Thompson *et al*. 1998).

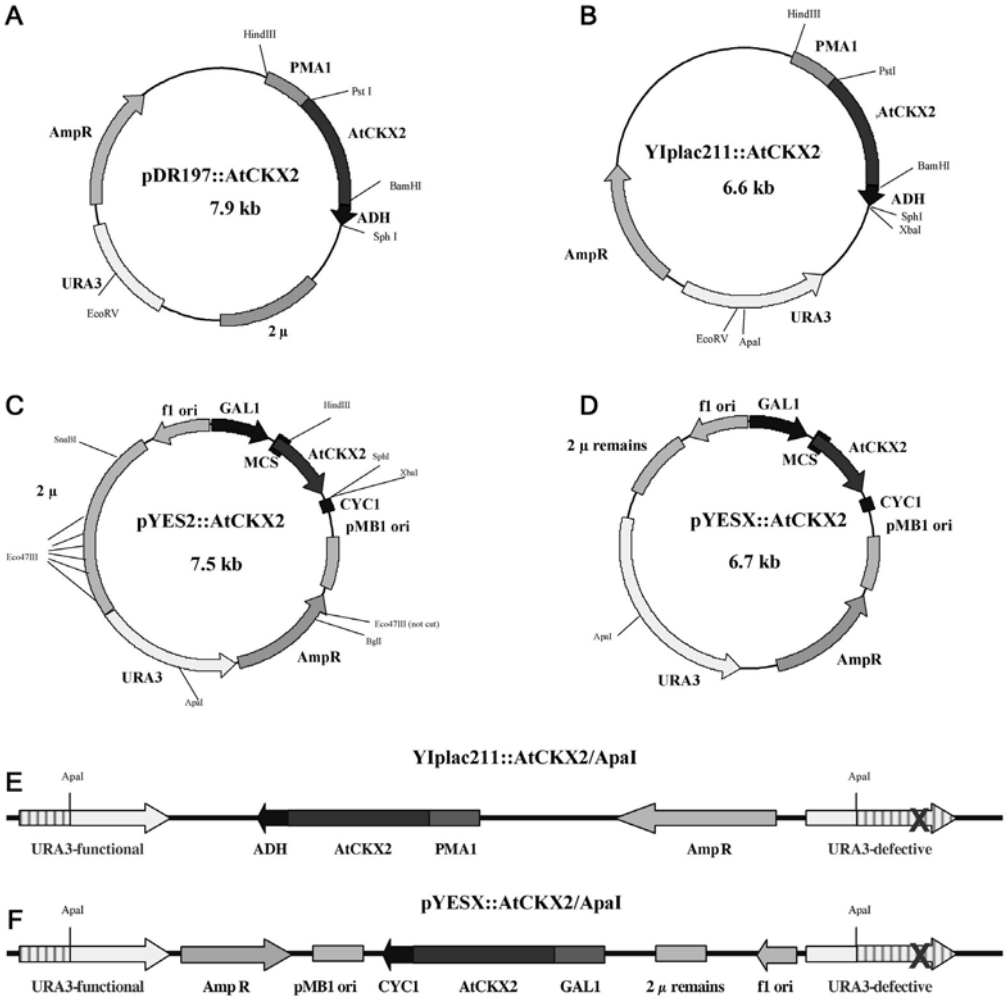

Fig. 1. Contructs used in this work: *A* - pDR197::AtCKX2, *B* - YIplac211::AtCKX2, *C* - pYES2::AtCKX2, *D* - pYESX::AtCKX2, *E* - YIplac211::AtCKX2/*Apa*I, *F* - pYESX::AtCKX2/*Apa*I. Abbreviations: ADH - alcohol dehydrogenase transcription terminator, AmpR - ampicilin resistance gene, AtCKX2 - *Arabidopsis thaliana* cytokinin dehydrogenase 2 cDNA (TAIR accession no. At2g19500), CYC1 - iso-1-cytochrome C gene transcription terminator, f1 ori - rescue site of ssDNA, GAL1 - galactose inducible promoter, MCS - multiple cloning site, PMA1 - plasma membrane ATPase promoter (constitutive), pMB1 ori - *Escherichia coli* origin of replication, URA3 - uracil autotrophy gene, 2 µ - *Saccharomyces cerevisiae* origin of replication.

Yeast genome integration construct with the constitutive promoter PMA1 (YIplac211::AtCKX2): To prepare the construct for gene integration, the gene cassette composed of the *PMA1* promoter, *AtCKX2* gene and *ADH* transcription terminator was cut out from pDR197::AtCKX2 with *Hind*III and *Sph*I and ligated into the yeast integration vector YIplac211. This vector does not replicate in yeast and carries a functional *URA3* gene that conveys uracil autotrophy to the used yeast strain. The plasmid construct YIplac211::AtCKX2 (Fig. 1*B*) was then linearized with *Apa*I within the *URA3* gene and used for integration into the yeast genome.

Yeast genome integration construct with inducible promoter GAL1 (pYESX::AtCKX2): The plasmid pYES2::AtCKX2 (Fig. 1*C*) obtained in previous work (Werner *et al*. 2001) was cleaved with blunt end restriction enzymes *Eco*47III and *Sna*BI to destroy the 2 µ yeast replication site. As found by restriction analysis, the Eco47III site 1961 was not digested under the conditions used. The main fragment was backligated to form pYESX::AtCKX2 (Fig. 1*D*). The plasmid construct was then linearized with *Apa*I within the *URA3* gene and used for the integration.

Gene integration into the yeast genome: The constructs linearized by *Apa*I as above were used to transform the uracil synthesis deficient yeast strain along with other plasmids as controls. The constructs cleaved within the *URA3* gene recombined within the defective *ura3* gene in the yeast genome and provided a functional copy of the gene along with integrating the insert of choice (Fig. 1*E*,*F*). Such transformants then acquired uracil autotrophy, used for the selection. Since gene recombi-

nation is a low efficiency process, much attention had to be paid to the transformation yield. Two independent methods were used, an electroporation (Thompson *et al*. 1998) and a heat shock (Gietz and Woods 2001) with 5 µg DNA per transformation. As controls, empty YIplac211 linearized with *Apa*I and circular pYESX::AtCKX2 were used. Only the heat shock method was efficient for the gene integration.

Screening of transformants: Several selected transformants of pDR197::AtCKX2, YIplac211::AtCKX2/*Apa*I and pYESX::AtCKX2/*Apa*I were picked up and grown for one day in 2 cm^3 of a selective medium (6.7 g dm^{-3} of yeast nitrogen base without amino acids, 2 % glucose, 0.1 M potassium phosphate buffer, pH 7.2) at 30 °C. The transformants pYESX::AtCKX2/*Apa*I were then induced overnight using the medium containing 2 % galactose in exchange for glucose. The yeast cells were removed by centrifugation at 5000 *g* for 5 min and the CKX activity was measured in the cell-free medium as follows.

CKX activity and protein assay: Cytokinin dehydrogenase activity was measured at 37 °C using spectrophotometric method based on the formation of a Schiff base between the enzymatic reaction product and *p*-aminophenol (Libreros-Minotta and Tipton 1995) in a reaction mixture containing a buffer, 0.15 mM substrate and 0.5 mM electron acceptor (Frébort *et al*. 2001). In standard assay conditions, 75 mM imidazol/HCl buffer, pH 7.0, iP as the substrate and 2,3-dimethoxy-5-methyl-*p*-benzoquinone (Q_0) as electron acceptor were used (Frébortová *et al*. 2004). For assessment of pH optimum a series of 100 mM buffers differing by 0.5 pH units was used: McIlvaine buffer, pH 4.0 - 7.5, imidazole/HCl buffer, pH 6.0 - 7.0, and Tris/HCl buffer pH 7.5 - 9.0. The pH optimum was measured with Q_0 and 2,6-dichlorophenol indophenol (DCPIP) as electron acceptors.

Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin as a standard.

Purification of AtCKX2: AtCKX2 was purified from the cell free medium of yeast transformed with pYESX::AtCKX2/*Apa*I. The yeast was cultivated at 25 °C in a medium (30 cm^3) containing 6.7 g dm^{-3} of yeast nitrogen base without amino acids, 0.1 M potassium phosphate buffer, pH 7.2, and 2 % galactose, with orbital shaking at 130 rpm. Additional 2 % galactose was added after 40 h of cultivation and the yeast were cultivated for next 24 h.

The cells were removed by centrifugation at 15 000 *g* and the pH of the cell free medium adjusted to pH 8.0 using 1 M Tris. The medium was filtered through 0.2 µm *MediaKap* filter (*Spectrum*, Rancho Dominguez, CA, USA) to remove residual yeast cells. The filtrate was concentrated to about 60 cm^3 by ultrafiltration on a *MiniKros Sampler Module* (*Spectrum*) with the cut-off 10 kDa, followed by another concentration step using a stirred ultrafiltration cell (*Millipore*, Bedford, MA, USA) equipped with a *YM 10* membrane (cut-off 10 kDa). The concentrated medium was dialyzed overnight against a 350-fold excess of 20 mM Tris/HCl buffer, pH 8.5, and concentrated to 1.5 cm^3 in an *Amicon Ultra-4* centrifugation device with cut-off 5 kDa (*Millipore*). The dialyzed and concentrated sample was purified on a *Mono Q HR 5/5* ion-exchange column (*Amersham Biosciences*, Pittsburgh, PA, USA) connected to *BioLogic Duo-Flow FPLC* system equipped with UV and conductivity detectors and *Model 2128* fraction collector (*Bio-Rad,* Hercules, CA, USA). After applying the sample, the column was washed with 2.7 cm^3 of 20 mM Tris/HCl, pH 8.5, followed by 2 cm^3 of the same buffer containing 5 % of 1 M NaCl. After the washing, the proteins were eluted using a linear gradient of 1 M NaCl (25 cm^3) from 5 to 25 %. The concentration of NaCl was then increased linearly to 100 % (1 cm^3) and strongly retained proteins were eluted with further 3 cm^3 of this buffer. Eluted proteins were collected in 1 cm^3 fractions. The fractions showing CKX activity were pooled and concentrated by ultrafiltration. The concentrated sample was applied onto a *Superdex 200 HR 10/30* column (*Amersham Biosciences*) pre-washed with 50 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl and the proteins were eluted with the same buffer. Active fractions were pooled and concentrated by ultrafiltration. Using the ultra-filtration device, the buffer was exchanged for 10 mM potassium phosphate buffer, pH 7.5, and the concentrated sample was applied onto a ceramic hydroxyapatite column *Bio-Scale CHT5-I* (*Bio-Rad*). The column was washed with 8.7 cm^3 of 10 mM potassium phosphate buffer, pH 7.5, and the proteins were then eluted by a linear gradient of 10 mM and 500 mM potassium phosphate buffers, pH 7.5 (25 cm^3, 0 - 50 %), followed by a short linear gradient to 100 % of 500 mM potassium phosphate buffer, pH 7.5 (2 cm^3) and an isocratic elution with the same buffer (6 cm^3). Active fractions were collected in two batches, one eluting during the isocratic wash after applying the sample and the second eluting during the first linear gradient. Both fractions were separately concentrated by ultrafiltration. The second fraction was further purified on a low pressure *Red Sepharose CL-6B* column (1.2 i.d. × 7.2 cm) (*Amersham Biosciences*). The buffer was exchanged for 20 mM Tris/HCl, pH 7.5 using the ultrafiltration device and the sample was applied onto the column connected to a *BioLogic LP* liquid chromatograph equipped with UV and conductivity detector and *Model 2128* fraction collector (*Bio-Rad*). CKX activity was not retained on this column, but washed out with 20 mM Tris/HCl, pH 7.5. Active fractions were collected, concentrated by ultrafiltration and stored at -20 °C.

SDS-PAGE and immunoblot analysis: SDS-PAGE was performed on a slab gel (10 %) in Tris-glycine running buffer according to Laemmli (1970). Before application, samples were heated at 95 °C for 10 min in the presence of 1 % SDS and 1 % 2-mercaptoethanol. Recombinant molecular mass standard mixture (*Sigma*) was used as a

marker. Gels were stained for proteins with Coomassie Brilliant Blue G-250. For the immunostaining procedure, proteins were transferred onto nitrocellulose membrane (0.45 μm) using a *MiniTrans* blot system (*Bio-Rad*). Proteins transferred to the membrane were blocked with 3 % gelatine in 20 mM Tris/HCl, pH 7.5, (TBS buffer) for at least 2 h. The membrane was twice washed with TBS buffer containing 0.05 % of *Tween-20* (*Tween*-TBS buffer) for 10 min, and then incubated for 2 h in TBS buffer containing 1 % gelatine and a rabbit polyclonal antibody raised against the C-terminal fragment of barley HvCKX1 (Galuszka *et al*., unpublished results). The membrane was washed twice with *Tween*-TBS buffer followed by incubation (2 h) with anti-rabbit IgG alkaline phosphatase conjugate in TBS buffer containing 1 % gelatine. After rinsing the membrane twice in *Tween*-TBS buffer and once in 100 mM Tris/HCl buffer, pH 9.5, containing 100 mM NaCl and 5 mM magnesium chloride (AP buffer), the membrane was stained for alkaline phosphatase. It was incubated in the dark in 20 cm^3 of AP buffer containing 3.5 mg of NBT and 7.5 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 5 min. Protein staining on the membrane was performed with Amidoblack 10B.

Results

Assortment of the expression system and growth conditions for AtCKX2 purification: Cytokinin dehydrogenase activity was found in all tested transformants after selection by uracil autotrophy. In all cases, the active protein was released into the growth medium. The optimization of protein expression was aimed at the highest total CKX activity and involved adjustments to the volume of the cultivation flask and the culture medium, the absorbance (A_{600}) of the yeast culture upon induction, and to the incubation time. The optimum volume was 30 cm^3 of culture medium in 100 cm^3 Erlenmeyer flasks. High CKX activities were obtained when the yeast cells pre-cultured on glucose as energy source were not diluted (A_{600} > 2.0) during the exchange of the culture medium (data not shown). The yeasts containing *AtCKX2* under the inducible promoter were also cultivated directly on a medium containing 2 % galactose, without pre-cultivation on glucose. In the case of pYES2::CKX2 transformed yeast, the CKX activity was only about 25 % of the value obtained with cultures pre-cultivated on glucose, but about 100 % with the pYESX::CKX2/*Apa*I transformant (Fig. 2*A*). Thus, eliminating the pre-cultivation on glucose enabled us to avoid laborious replacement of the culture media and reduced the cost of cultivation. Individual transformants were grown at 30 °C with orbital shaking at 130 rpm in 4 × 30 cm^3 of culture media until no further increase in CKX activity was observed. The culture media were 100-fold concentrated and total and specific CKX activities were evaluated (Fig. 2*A*). Except for pYES2::CKX2 transformed yeast, there were no major differences among the yeast transformants in CKX activity per cm^3 of culture medium or in their specific activities. The pYESX::CKX2/*Apa*I transformant showed both high CKX activity per cm^3 of culture medium and a high specific activity and was chosen for further studies.

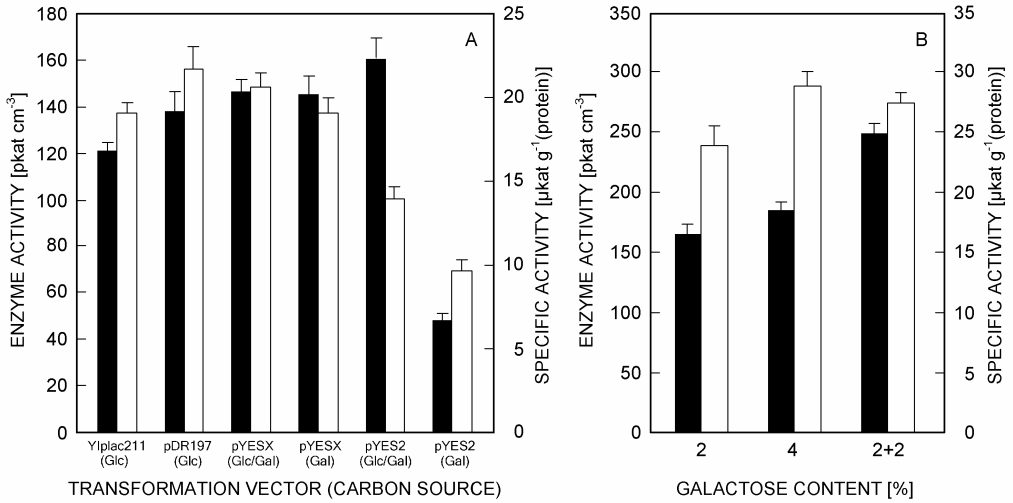

Fig. 2. Expression of AtCKX2 in *Saccharomyces cerevisiae*. *A* - the enzyme activity (*black bars*) and specific activity (*white bars*) in supernatants of yeast transformed with different expression vectors carrying the *AtCKX2* gene (energy source: Glc - 2 % glucose, Glc/Gal - 2 % glucose replaced with 2 % galactose for the induction of expression, Gal - 2 % galactose), *B* - the effect of initial galactose concentration on the activity of pYESX::AtCKX2/*Apa*I transformant (in case of the 2+2 sample, the medium was supplemented with additional 2 % of galactose after 40 h of growth). All cultures were grown on yeast nitrogen basal medium without amino acids at 30 °C (*A*) or 25 °C (*B*) with orbital shaking at 130 rpm to stationary phase (A_{600} > 2.5). Each transformant was cultivated in duplicate in two independent experiments. Means with SD.

Table 1. Purification of recombinant AtCKX2 from *Saccharomyces cerevisiae* pYESX::CKX2/*Apa*I.

Purification step	Total protein [mg]	Total activity [nkat]	Specific activity [μkat g^{-1}]	Recovery [%]	Purification [fold]
Concentrated cell-free medium	10.5	321.9	30.6	100	1.0
Mono Q	3.1	230.4	74.3	72	2.4
Superdex 200 HR	2.7	229.8	85.1	71	2.8
Hydroxyapatite	0.7	42.9	61.3	13	2.0
Red Sepharose	0.4	35.1	87.8	11	2.9

Specific CKX activity increased by 25 % when the pYESX:CKX2/*Apa*I transformed yeasts were cultivated at 25 °C instead of 30 °C (compare data in Fig. 2*A,B*). Growth on a medium containing 4 % galactose showed only a slight increase in CKX activity compared to the growth on 2 % galactose, but a supplementation of the 2 % medium with additional 2 % of galactose after 40-h growth, when no further increase in CKX activity was detected, led to enhanced production of AtCKX2 protein (Fig. 2*B*).

Purification of AtCKX2: AtCKX2 was purified from the cell-free medium of yeast transformed with pYESX::CKX2/*Apa*I and grown at 25 °C in the presence of 2 % galactose. The enzyme production was further enhanced by an addition of 2 % galactose after 40-h growth. The pH of the cell free medium was adjusted to 8.0, which stabilizes the protein (the enzyme is stable for at least two weeks at +4 °C). The medium was concentrated by ultrafiltration as the ammonium sulfate precipitation was not effective (less than 40 % of the activity precipitated with 80 % saturation of ammonium sulfate). Enzyme purification was attempted by a number of different strategies. The optimized purification procedure is summarized in Table 1. The enzyme was purified by ion-exchange, gel filtration, hydroxyapatite and *Red Sepharose* chromatographies (Fig. 3). The chromatography on hydroxyapatite resulted in a decrease of specific activity and low recovery (Table 1). This was due to the fact that the activity separated into two peaks, one eluting during an isocratic wash at the start of the chromatographic run and the other eluting with the gradient (Fig. 3C). The former fraction contained 1.4-fold more CKX activity than the latter, but SDS-PAGE showed that the first fraction contained only small amount of the protein with molecular mass corresponding to AtCKX2 in addition to a protein smear of 75 - 100 kDa. CKX was not retained on *Red Sepharose*, but contaminating proteins were removed at this step (Table 1).

The purity of the enzyme was examined by SDS-PAGE, where it migrated as a sharp band corresponding to molecular mass of approximately 61 kDa accompanied by weaker fuzzy protein band of molecular mass between 75 and 100 kDa (Fig. 4*A*). Both protein bands were detected with polyclonal antibody raised against barley HvCKX1, suggesting that the fuzzy band represented an aggregated form of CKX (Fig. 4*A*). The specific activity of purified enzyme was 87.8 μkat g^{-1} with iP as the substrate. The recovery of purification procedure was 11 %, which corresponds to 0.2 mg of purified AtCKX2 from 1 dm^3 of the yeast culture.

AtCKX2 properties: The purified protein showed a molecular mass of 61 kDa as determined by SDS-PAGE. This value is higher than the 53.3 kDa predicted from amino acid sequence (without the putative 22 amino acid secretion signal peptide, http://www.cbs.dtu.dk/ services/ SignalP/). This difference can be attributed to glycosylation of the AtCKX2 protein, which contains

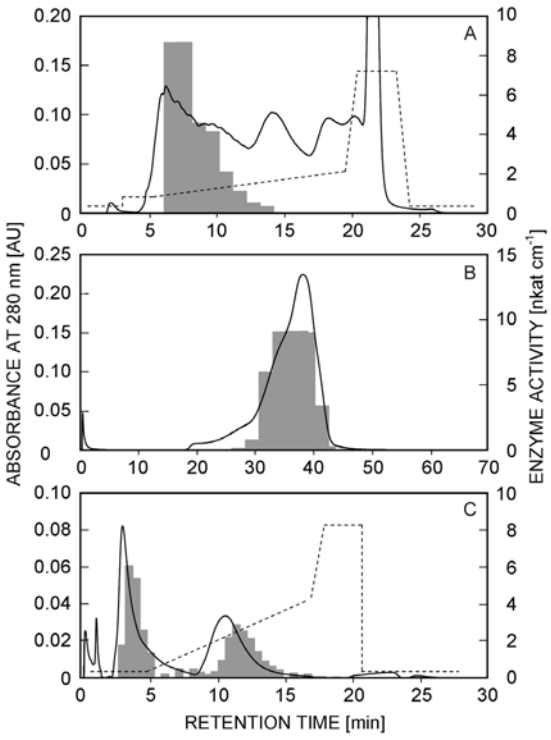

Fig. 3. FPLC purifications of recombinant AtCKX2. The chromatographic charts show the absorbance at 280 nm (solid line), enzyme activity (*grey bars*) and gradient shape, where applied (*broken line*): *A* - Mono Q (20 mM Tris/HCl, pH 8.5, a gradient of 0 - 100 % of 1 M NaCl, flow rate 1 cm^3 min^{-1}), *B* - Superdex 200 HR (50 mM Tris/HCl buffer, pH 7.5, 0.15 M NaCl, flow rate 0.4 cm^3 min^{-1}), *C* - ceramic hydroxyapatite Bio-Scale CHT I (potassium phosphate buffer, pH 7.5, a gradient of 10 - 500 mM, flow rate 2 cm^3 min^{-1}). For further details see Materials and methods.

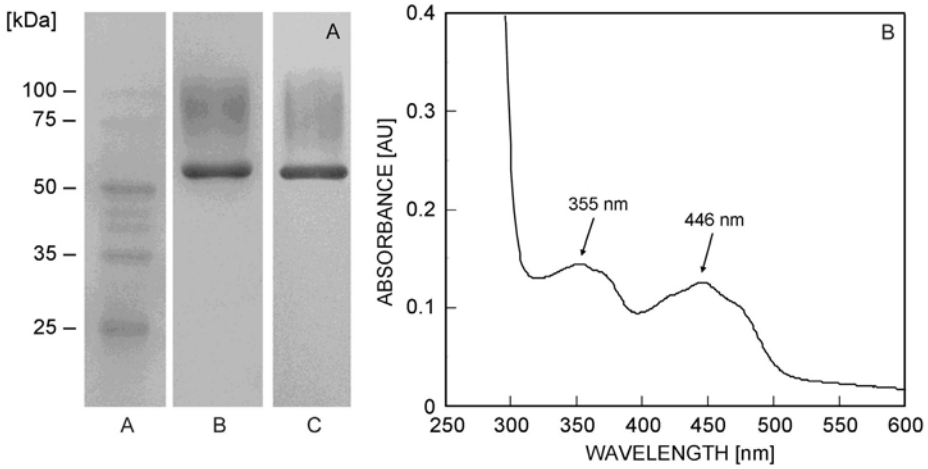

Fig. 4. Properties of purified recombinant AtCKX2. *A* - SDS-PAGE. *Lane A*: molecular mass standards; *lane B*: purified AtCKX2 stained with Coomassie Brilliant Blue; *lane C*: immunoblot analysis of purified AtCKX2 stained with antibody raised against barley HvCKX1 (see Materials and Methods for further experimental details). *B* - absorption spectra of AtCKX2 (10.7 μM) in 20 mM Tris/HCl, pH 7.5.

three presumable N-glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc). The purified enzyme was of yellow colour and showed the typical absorption spectra of flavoproteins, with absorption maxima at 355 and 446 nm (Fig. 4*B*).

The activity of AtCKX2 was investigated over a pH range from 4 to 9. The optimum pH was dependent on the electron acceptor used in the CKX assay, and was 7.0 to 7.5 with Q_0 and 8.0 with DCPIP (data not shown).

Table 2. Activity of AtCKX2 with various cytokinins. Measured according to Frébort *et al*. (2001) in 75 mM imidazole/HCl, pH 7.0, with 150 μM substrate and 500 μM Q_0 as the electron acceptor (n.d. - not detected).

Cytokinin	Specific activity [μkat g^{-1}]	Relative activity [%]
iP	86.8	100.0
iPR	65.2	75.1
iP9G	11.1	12.8
iPRMP	3.7	4.3
t-Z	250.9	289.1
t-ZR	128.2	147.7
Z9G	6.9	7.9
DHZ	n.d.	-
MiP	0.8	0.9
Kinetin	2.5	2.9
BAP	0.3	0.4
o-T	0.1	0.1
m-T	0.2	0.2
p-T	0.2	0.2

The substrate specificity was studied with different isoprenoid cytokinins, including nucleosides and glucosides, as well as aromatic cytokinins (Table 2). AtCKX2 reacted best with *t*-Z and *t*-ZR, followed by iP and iPR. iP-ribotide showed only 4 % of the activity of free iP. Low activity was found with 9-glucosides (12.8 and 7.9 % of iP value for iP9G and Z9G, respectively), with a N^6-methylated cytokinin analogue, MiP (0.9 %), and with synthetic cytokinin kinetin (2.9 %). AtCKX2 reacted at very low rates also with aromatic cytokinins, BAP (0.4 %), *o*-T (0.1 %), *m*-T (0.2 %) and *p*-T (0.2 %), similarly to maize ZmCKX1 (Frébortová *et al*. 2004). Dihydrozeatin was not a substrate of AtCKX2.

Table 3**.** Reactivity of AtCKX2 with different electron acceptors ($^aCu^{II}$/imidazole complex functions as the electron acceptor). Measured in 75 mM imidazole/HCl, pH 7.0, with 150 μM iP as a substrate and 500 μM electron acceptor (n.d. - not detected).

Acceptor added	Specific activity [μkat g^{-1}]	Relative activity [%]
None (O_2)	1.8	2.1
Q_0	87.7	100.0
1,4-naphthoquinone	248.6	283.3
Vitamin K_3	10.6	12.1
Vitamin K_1	n.d.	n.d.
Duroquinone	6.4	7.3
Potassium ferricyanide	2.5	2.8
$CuCl_2$[a]	13.4	15.3
DCPIP	179.1	204.1

The K_m value for iP as substrate and Q_0 as electron acceptor was determined to be 1.5 μM.

AtCKX2 reacted poorly when oxygen as the sole electron acceptor was present (Table 3). The reaction rate was greatly enhanced by *p*-quinones 1,4-naphthoquinone (140-fold) and Q_0 (50-fold), as well as by other synthetic electron acceptors such as DCPIP (100-fold) and Cu^{2+}/imidazole complex (7.5-fold). These data are in agreement with the classification of the cytokinin degrading enzyme as dehydrogenase (EC 1.5.99.12) and with the properties of the best studied CKX, ZmCKX1 from maize (Frébortová *et al*. 2004, Kopečný *et al*. 2005).

Discussion

Cytokinin degradation in plants is regulated by CKX enzymes, encoded by small gene families (Schmülling *et al*. 2003). In *Arabidopsis thaliana* the family consists of seven genes, *AtCKX1*-*AtCKX*7 (Schmülling *et al*. 2003), which show differential expression throughout plant development (Werner *et al*. 2003). In addition to different gene expression patterns, the proteins appear to have different subcellular localizations and biochemical properties (Schmülling *et al*. 2003, Werner *et al*. 2003, Galuszka *et al*. 2007). The purification of individual proteins from plant material does not seem feasible not only because of the simultaneous presence of several CKX proteins in plant tissues but also due to their extremely low content. The same is true for CKX from other plants, *e.g*. 100 μg of ZmCKX1 was purified from 40 kg of maize kernels, and 100 and 250 μg of CKX were purified from 1 kg of wheat and barley grains, respectively (Morris *et al*. 1999, Galuszka *et al*. 2001). Efficient purification of large quantities of homogenous enzyme was achieved only after cloning the *ZmCKX1* gene and its expression in yeast host (Houba-Herin *et al*. 1999, Morris *et al*. 1999, Bilyeu *et al*. 2001, Kopečný *et al*. 2005). ZmCKX1 was purified by one step gel chromatography from a supernatant of methylotrophic yeast *Pichia pastoris* (Bilyeu *et al*. 2001) and by gel and ion exchange chromatographies from supernatant of *Yarrowia lipolytica* (Kopečný *et al*. 2005).

Out of the seven *Arabidopsis* CKX enzymes, AtCKX*2* appears to be the most abundant secreted protein (Schmülling *et al*. 2003, Galuszka *et al*. 2007). *AtCKX2* has been previously cloned into pYES2 multicopy plasmid under the control of the *GAL1* promoter and shown to give an active protein in yeast *S. cerevisiae* (Werner *et al*. 2001). In this work, the *AtCKX2* gene was integrated into the yeast chromosome and its constitutive and inducible expressions were studied to select the best system for the production of recombinant AtCKX2. During the course of the study we also evaluated the possibility of preparing His-tagged AtCKX2 using pYES2.1/V5 His-TOPO expression vector to facilitate the protein purification. However, the His-tagged protein was not retained on a nickel metal-affinity resin, probably due to the inaccessibility of the His-tag positioned at the C-terminus of the enzyme. The C-terminus of a related protein, maize ZmCKX1, is indeed deeply buried inside the protein (Malito *et al*. 2004). The expression in *E. coli* was also tested but the presence of active protein was not detected.

The expression system based on the pYESX:AtCKX2/*Apa*I vector produced under optimum conditions about 2 to 3 mg of the recombinant protein per dm^3 of culture. Although there were not too many contaminating proteins as the first column purification yielded a protein of about 80 % purity, judging from specific activities (see Table 1; note that the protein content in the cell-free medium may be underestimated as the yeast produces low molecular proteins and peptides that react poorly with Coomassie Brilliant Blue), the removal of remaining impurities proved exceptionally difficult as the AtCKX2 protein exhibited low polarity that eventually resulted in fractional formation of an aggregate (see Fig. 4*A*). Several other purification strategies, such as glycoprotein affinity binding on ConA-Sepharose 4B, hydrophobic chromatography on Hema-Bio Phenyl and Resource Phenyl and chromatofocusing on Mono P were ineffective. These problems resulted in a final purification yield of only 11 % of the pure recombinant protein. Purification problems due to low polarity of CKX have been previously encountered with the enzymes from barley and wheat (Galuszka *et al*. 2001), but not for the recombinant ZmCKX1 (Bilyeu *et al*. 2001, Kopečný *et al*. 2005), so this feature may be closely related to the amino acid sequence, the structure of the protein and/or posttranslational modifications.

The recombinant AtCKX2 showed preference for isoprenoid cytokinins, with *t*-Z and *t*-ZR being best substrates (Table 2). The reactivity with aromatic cytokinins was very low. The findings are in general agreement with results obtained with a secreted enzyme from maize, ZmCKX1 (Bilyeu *et al*. 2001, Frébortová *et al.* 2004, Kopečný *et al*. 2005). The K_m value determined for iP (1.5 μM) is in agreement with ZmCKX1, although this value should be considered apparent as it strongly depends on the concentration ratio of substrate, electron acceptor and the enzyme, due to the ternary mechanism of two-substrate reaction (Popelková *et al*. 2006, Frébort *et al*. 2001). As for ZmCKX1, the oxygen is a poor electron acceptor (Table 3). The reaction rate is enhanced by the synthetic dye DCPIP, as well as *p*-quinones such as 1,4-naphthoquinone and an ubiquinone analogue Q_0. The identity of the electron acceptor *in vivo* remains to be elucidated.

The specific activity of the enzyme was 87.8 μkat g^{-1} (with iP and Q_0) that corresponds to k_{cat} of 5.4 s^{-1}, which is much lower than about 140 s^{-1} found for ZmCKX1 (Frébortová *et al*. 2004), but still higher than for the enzymes from barley and wheat (Galuszka *et al*. 2001). We expect that in accordance with their tissue distribution and subcellular localization, different isoenzymes would act on different cytokinin substrates with different velocity and specificity thus fulfilling a role of controlling agents in the fine tuning of local cytokinin concentration that must be synchronized with other components of the cytokinin metabolism, transport and signaling (Sakakibara *et al*. 2006), in order to stimulate the proper physiological response.

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Trigiano, R.N., Gray, D.J. (ed.): **Plant Development and Biotechnology.** - CRC Press, Boca Raton - London - New York - Washington 2005. 358 pp. ISBN 0-8493-1614-6.

Editors of this book are excellent experts in plant biotechnologies: Prof. Robert N. Trigiano from the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville, USA, and Dr. Dennis J. Gray from the University of Florida, IFAS, Horticulture Department. The presented book covers the major issues in the field of plant development and biotechnologies and discusses new techniques and opportunities. The book employs an informal style to address the main aspects of development and biotechnology with minimal references, without sacrificing information or accuracy. The book contains 29 chapters and their division into the five sections was not necessary. Both Section I and Section II contain only one chapter "Introduction" and "History of Plant Tissue Culture", respectively. These two chapters and the Chapter 3 ("Getting Started with Tissue Culture: Media Preparation, Sterile Technique and Laboratory Equipment") from Section III ("Supporting Methodologies") are appropriate to the beginners in the field of plant tissue culture. For this group of researchers a short summary of plant growth regulators often used in plant tissue cultures may be also important (Chapter 8). To the Section III further belong chapters dealing with histological techniques (Chapter 4) and photographic methods (Chapter 5). Very useful is Chapter 9 presenting software and databases for analyzing nucleic acid and protein sequences. Following section IV ("Propagation and Development Concepts") offers the articles connecting with the micropropagation and organogenesis (Chapters 12 and 13) and somatic embryogenesis (Chapters 14 and 15) and presenting also the molecular aspects. "Crop Improvement Techniques" is the name of Section V. We can found here methods for isolation of protoplasts and regeneration of plants from the protoplasts (Chapter 16), haploid cultures (Chapter 17), embryo rescue procedure (Chapter 18), and germplasm preservation (Chapter 23). The main topic of this section is genetic engineering technologies (Chapters 19 to 22) describing three major DNA transfer methods: *Agrobacterium*-mediated transformation, direct protoplast-mediated DNA transfer and microprojectile bombardment. Following chapter discuss the problems with public view to cultivation of genetically modified (GM) plants, the food safety of GM plant products and ecological interaction between GM plants and wild plants. At the end of this section we can found a short chapter about utilization of *in vitro* culture for production of pharmacologically important secondary metabolites. The last section IV ("Special topics") contains five chapters. Two chapters deal with plant pathology: development of disease-resistant genotypes of crop plants (Chapter 25) and diagnostic methods for detection and identification of viral and bacterial plant pathogens (Chapter 28). Very unusual, but interesting, is the Chapter 27 ("Commercial Laboratory Production") giving the advice how to utilize micropropagation as a practical and cost-effective production tool. This chapter describes some of the basic steps involved in plant micropropagation, discusses the practical aspects of the micropropagation business, and gives some examples of how and why micropropagation is being now used by growers.

Every chapter starts with brief concept and ends by list of cited literature and suggested readings. The text is complemented by tables, figures, schemes and black and white photographs. I can suggest this book to all research teams started with plant tissue culture development, micropropagation, genetic plant transformation or other *in vitro* plant biotechnologies.

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