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Biochemical Characterization of Cytokinin Oxidases/Dehydrogenases from *Arabidopsis thaliana* Expressed in *Nicotiana tabacum* L.

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Abstract Transgenic tobacco plants overexpressing single *Arabidopsis thaliana* cytokinin dehydrogenase (CKX, EC 1.5.99.12) genes *AtCKX1*, *AtCKX2*, *AtCKX3*, *AtCKX4*, *AtCKX5, AtCKX6*, and *AtCKX7* under the control of a constitutive 35S promoter were tested for CKX-enzymatic activity with varying pH, electron acceptors, and substrates. This comparative analysis showed that out of these, only AtCKX2 and AtCKX4 were highly active enzymes in reaction with isoprenoid cytokinins (N^6-(2-isopentenyl) adenine (iP), zeatin (Z)) and their ribosides using the artificial electron acceptors 2,6-dichlorophenol indophenol (DCPIP) or 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0). Turnover rates of these cytokinins by four other AtCKX isoforms (AtCKX1, AtCKX3, AtCKX5, and AtCKX7) were substantially lower, whereas activity of AtCKX6 was almost undetectable. The isoenzymes AtCKX1 and AtCKX7 showed significant preference for cytokinin glycosides, especially N^6-(2-isopentenyl)adenine 9-glucoside, under weakly acidic conditions. All enzymes preferentially cleave isoprenoid cytokinins in the presence of an electron acceptor, but aromatic cytokinins are not resistant and are degraded with lower reaction rates as well. Cytokinin nucleotides, considered as resistant to CKX attack until now, were found to be potent substrates for some of the CKX isoforms. Substrate specificity of AtCKXs is discussed in this study with respect to the structure of the CKX active site. Further biochemical characterization of the AtCKX1, AtCKX2, AtCKX4 and AtCKX7 enzymes showed pH-dependent activity profiles.

Keywords Activity staining · *Arabidopsis thaliana* · Cytokinin oxidase/dehydrogenase · pH optimum · Substrate specificity · *Nicotiana tabacum*

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Introduction

The plant hormone cytokinin is selectively inactivated by oxidative cleavage of its side chain. The enzyme catalyzing this degradation has been named cytokinin oxidase for a long time (Mok and Mok 2001), but recent findings indicate that the enzyme works more efficiently with an electron acceptor other than oxygen (Galuszka and others 2001; Laskey and others 2003; Frébortová and others 2004; Popelková and others 2006). Therefore, the enzyme was reclassified and is now called cytokinin dehydrogenase (CKX) or cytokinin oxidase/dehydrogenase. CKX removes the isoprenoid side chain of N^6-(2-isopentenyl)adenine (iP) and zeatin (Z), converting them to adenine and the corresponding unsaturated aldehyde (Galuszka and others 2001;

Frébortová and others 2004). Cytokinin oxidase/dehydrogenase is a flavoprotein containing covalently bound FAD molecule as a cofactor (Galuszka and others 2001; Laskey and others 2003; Bilyeu and others 2001). Genome-wide studies revealed that in different higher plant species, CKX proteins are encoded by small gene families with a varying number of members (Werner and others 2006). *CKX* genes were cloned from a variety of plant species, for example, *Zea mays* (Houba-Hérin and others 1999; Morris and others 1999; Massonneau and others 2004), *Arabidopsis thaliana* (Bilyeu and others 2001; Werner and others 2001), *Dendrobium* sp. (Yang and others 2002, 2003), and recently also from *Hordeum vulgare* (Galuszka and others 2004). However, a comparative study of biochemical properties of individual CKX proteins from a complete family has not been accomplished yet.

In *Arabidopsis thaliana*, seven distinct CKX-encoding genes (*AtCKX1-AtCKX7*) were identified. N-terminal signal peptides of the AtCKX proteins indicate that AtCKX2, AtCKX4, AtCKX5, and AtCKX6 are possibly targeted to the plant secretory pathway, which was experimentally confirmed by study of AtCKX2-GFP localization (Werner and others 2003). AtCKX2 is also secreted when expressed in the yeast host cells (Bilyeu and others 2001; Werner and others 2001). AtCKX1- and AtCKX3-GFP fusion proteins were targeted to vacuoles, whereas the AtCKX7 protein lacks any obvious targeting sequence and is presumably localized to the cytosol (Werner and others 2003). Amino acid sequence comparison revealed that individual CKX proteins from *Arabidopsis* share conserved regions of high homology (e.g, FAD-binding domain), but their sequences outside of these domains display a strong divergence (Popelková and others 2004). This might be suggestive of a functional diversification reflected by different biochemical characteristics of paralogous AtCKX proteins.

In this work we examined enzymatic properties of all seven enzymes from *Arabidopsis thaliana* expressed in transgenic tobacco plants. *In vitro*, all isoenzymes prefer dehydrogenase mode of action with electron acceptors 2,6-dichlorophenol indophenol (DCPIP), ubiquinone precursor 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0), or other similar quinonic structures. Values of turnover rates and substrate specificity of individual enzymes are presented and discussed with respect to the structure and amino acid composition of their active sites predicted by molecular modeling with the available crystal structure of ZmCKX1 from maize (Malito and others 2004) and AtCKX7 (Wesenberg and others 2005) as templates. The biochemical data are accompanied by a histochemical localization of activity of AtCKX1, AtCKX2, and AtCKX4.

Materials and Methods

Chemicals

N^6-(2-iso-pentenyl)adenine 7-glucoside (iP7G), N^6-(2-iso-pentenyl)adenine 9-glucoside (iP9G), N^6-(2-iso-pentenyl)adenine 9-riboside-5′-monophosphate (iPRMP), *trans*-zeatin (Z), *trans*-zeatin 9-riboside (ZR), *cis*-zeatin (cisZ), *trans*-zeatin 9-glucoside (Z9G), *trans*-zeatin O-glucoside (ZOG), *trans*-zeatin O-acetyl (AcZ), dihydrozeatin (DHZ), *o*-topolin (oT), *m*-topolin, (mT), *p*-topolin (pT), and *p*-methoxytopolin (MepT) were from OlChemIm (Olomouc, Czech Republic). 2,6-Dichlorophenol indophenol (DCPIP) was from LOBA Feinchemie (Fischamend, Austria). 1,4-Naphthoquinone was from BDH Chemicals (Poole, England) and 2,3,5-triphenyl tetrazolium, methylene blue, and potassium ferricyanide were from Lachema (Brno, Czech Republic). N^6-(2-isopentenyl)adenine (iP), N^6-(2-isopentenyl)adenine 9-riboside (iPR), N^6-benzyladenine (BA), N^6-benzyladenine 9-riboside (BAR), kinetin (K), phenazine methosulfate (PMS), phenylmethylsulfonyl fluoride (PMSF), nitroblue tetrazolium (NBT), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0), NAD^+, $NADP^+$, salicylic acid, acetyl CoA, cytochrome *c*, methylthiazolyldiphenyl-tetrazolium, oxidized glutathione, syringaldehyde, duroquinone, 3′,5′-dimethoxy-4′-hydroxyacetophenone, vitamin K_3 (menadione, 2-methyl-1,4-naphthoquinone), vitamin K_1 (phylloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone), isovaleraldehyde, and other chemicals were from Sigma (St. Louis, MO, USA).

Plant Material

Construction of transgenic tobacco plants overexpressing *Arabidopsis AtCKX1*, *AtCKX2*, *AtCKX3*, and *AtCKX4* genes was reported previously (Werner and others 2001). Binary plasmids harboring *AtCKX5* (At1g75450) and *AtCKX6* (At3g63440) under the control of a 35S promoter were described earlier (Werner and others 2003). The cloning of AtCKX7 will be described in detail elsewhere (Werner and others, unpublished). The binary vectors were transformed into *Agrobacterium tumefaciens* GV3101 (pMP90) and *Nicotiana tabacum* L. cv. Samsun NN explants were transformed and regenerated according to the standard protocol (Horsch and others 1985). At least ten independently derived transgenic lines were selected for each construct. Transgenic tobacco lines showing the strongest phenotype or highest CKX activity (clones AtCKX1-50, AtCKX2-38, AtCKX3-9, AtCKX4-27, AtCKX5-15, AtCKX6-53, and AtCKX7-12) were selected for the experiments. Plants were grown in a growth chamber

with 12-h light/dark cycles at 24°C and leaf material was harvested before flower induction.

CKX Activity Assay

Plant samples (young upper leaves) were cut to pieces, powdered in liquid nitrogen using a hand mortar, and extracted with twofold excess (v/w) of the extraction buffer (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 passed through a Sephadex G-25 column (50 cm × 2.5 cm i.d.), equilibrated with extraction buffer and 1 mM PMSF to remove low-molecular-weight compounds, and concentrated using an ultrafiltration cell (Amicon, Danvers, MA, USA) equipped with a 10-kDa cutoff membrane XM 100 (Sigma).

The activity was measured using a modified endpoint method described earlier (Frébort and others 2002). For activity screening in plant organs, substrate specificity, and pH profile measurements, the samples were incubated in a reaction mixture (total volume of 0.6 ml in 1.5-ml tube) that consisted of 100 mM McIlvaine buffer, pH 4.0-8.5, 500 μM DCPIP or Q_0, 250 μM substrate, and concentrated transgenic tobacco extract in volumes of 5-75 μl. Incubation time at 37°C was 2-8 h, depending on the enzyme activity. All substrates were dissolved in dimethylsulfoxide, final concentration 5% in the reaction mixture.

To screen for other active electron acceptors, the following compounds at the particular final concentrations were used in the reaction mixture instead of DCPIP/Q_0: 1 mM duroquinone, 3′,5′-dimethoxy-4′-hydroxyacetophenone, methylthiazolyldiphenyl-tetrazolium, NBT, 2,3,5-triphenyl tetrazolium, oxidized glutathione, NAD^+, $NADP^+$, methylene blue, and salicylic acid; 0.25 mM acetyl CoA; 0.25 mM 1,4-naphthoquinone, vitamin K_1, vitamin K_3, syringaldehyde, and PMS; 0.1 mM potassium ferricyanide; and 0.05 mM cytochrome *c*. To test the oxidase mode of the enzymatic reaction, the electron acceptor was substituted for by water in the reaction mixture.

The enzymatic reaction was stopped after incubation by adding 0.3 ml of 40% trichloroacetic acid, 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% trichloroacetic acid) was added to the supernatant followed by immediate (within 3 min) scanning of the absorption spectrum from 300 to 700 nm to determine the concentration of product Schiff base specific for the given substrate [ε_{352} = 15.2 mM^{-1} cm^{-1} for iP, iPR, iPRMP, iP7G, and iP9G; ε_{352} = 3.4 mM^{-1} cm^{-1} for Z, ZR, Z9G, and AcZ; ε_{380} = 4.0 mM^{-1} cm^{-1} for K; ε_{365} = 0.3 mM^{-1} cm^{-1} for BA and BAR; ε_{380} = 1.5 mM^{-1} cm^{-1} for pT; ε_{330} = 4.3 mM^{-1} cm^{-1} for oT; ε_{315} = 2.4 mM^{-1} cm^{-1} for mT; and ε_{380} = 2.5 mM^{-1} cm^{-1} for MepT (Frébort and others 2002)].

cisZ and the other substrates (DHZ, ZOG), where the production of Schiff base with 4-aminophenol was not detected, were also tested by a continuous method based on bleaching of DCPIP (Laskey and others 2003). The reaction mixture was the same as for the above-mentioned endpoint method with the exception of DCPIP, which was added in the concentration of 0.15 mM. The reaction was started by addition of substrate and a decrease in absorbance at 600 nm was continuously monitored for 1 h. A blank without substrate was run in a parallel experiment to eliminate nonspecific bleaching of DCPIP caused by other redox compounds in the enzyme preparation.

For determination of specific activities, protein content in the samples was assayed according to Bradford (1976) with bovine serum albumin used as a standard.

Histochemical Localization of Enzymatic Activity

Leaves and roots of the tobacco plants were cut into 2-cm segments. Each segment was sandwiched between two pieces of a longitudinally cut *Sambucus* pith, sliced into 20-40-μm cross sections by a sliding microtome (AllMikro, Braunschweig, Germany). Sectioned samples were then immersed into the 0.15 mM Tris/HCl buffer, pH 8.0. Sections were then stained for CKX activity using a previously published protocol (Galuszka and others 2005) based on formation of a purple NBT formazan. The cross sections were immersed in a staining solution containing 0.4 mM substrate (iP), 0.15 mM PMS, and 0.75 mM NBT in 0.15 M Tris/HCl buffer, pH 8.0, incubated for 1-14 h at room temperature in the dark, and examined for purple product formation. The control experiment in which the sections were immersed in the staining solution without substrate was carried out.

After incubation, the sections were washed with 0.15 M Tris/HCl, buffer, pH 8.0, placed in a drop of 50% glycerol (w/v) on a glass mount, and viewed and photographed using a Model BX50 light microscope (Olympus, Tokyo, Japan) equipped with a CoolSNAP camera (Roper Scientific, Tucson, AZ, USA) connected to a PC. Due to light sensitivity of the staining reagents, prolonged light exposure of the viewed samples was avoided.

Computer Analysis of Amino Acid Sequence and Protein Structure

Protein sequence alignment was performed with the software BioEdit 7.0.4.1 using the ClustalW algorithm (Hall 1999). Structural alignment of ZmCKX1 in complex with

iP (PDB entry 1W1Q) and AtCKX7 (PDB entry 2EXR) was done with Open-Source PyMOL 0.99rc2 (DeLano Scientific LLC, South San Francisco, CA, USA).

Results

Tobacco Plants Expressing Individual *AtCKX* Genes

Because of the large homology shared among the members of the *Arabidopsis* CKX family, overlaps in expression of the genes, and low content of the CKX proteins in the wild-type tissues, it is virtually impossible to purify individual isoforms directly from a given wild-type plant species and study their biochemical properties, including substrate specificity, kinetic parameters, and pH optimum. Furthermore, the expression of recombinant CKX proteins in *E. coli* is host-toxic (our unpublished results). Therefore, as an alternative source of recombinant protein, we have utilized seven transgenic tobacco lines, each overexpressing a single member of the *Arabidopsis CKX* gene family under the control of constitutive 35S promoter (Gatz and others 1992). By utilization of such a plant heterologous expression system, one can see advantages in correct post-translational modifications, e.g., glycosylation which seems to play an important role in regulation of CKX protein activity (Motyka and others 2003).

Transgenic lines expressing *AtCKX1*, *AtCKX2*, *AtCKX3*, and *AtCKX4* are characterized by a high level of transgene expression, reduced cytokinin content, and typical phenotypic traits related to cytokinin deficiency (Werner and others 2001). In addition, we generated transgenic lines expressing constitutively *AtCKX5*, *AtCKX6*, and *AtCKX7* genes and characterized these plants according to their phenotype, transgene expression level, and/or CKX-specific activity. For CKX protein extraction, the line with the strongest phenotype was selected from several independent transformations. Total CKX activity correlated well with the strength of phenotype when different plants transformed with the same construct were tested. Compared to control plants, transgenic plants expressing *AtCKX5* exhibited strongly retarded shoot growth similar to the growth of *AtCKX1* and *AtCKX3* expressers, which indicates that a high expression level was achieved. Thus, similar to the study with *Arabidopsis* plants (Werner and others 2003), overproduction of AtCKX1, AtCKX3, or AtCKX5 in tobacco leads to stronger phenotype alterations than the overexpression of other AtCKX family members. This result suggests that the former three AtCKX proteins might share similar biochemical features. Plants overexpressing *AtCKX6* or *AtCKX7* showed only subtle changes in shoot development; therefore, more than 15 independent lines were screened for an increased CKX-specific activity (data not shown), and the plants showing the highest activity in comparison to the wild type were selected for further experiments. Specific activities of overexpressed AtCKX enzymes were stable during tobacco development from the stage of 2-week-old seedlings to senescence (data not shown).

A method based on using the artificial electron acceptor DCPIP or Q_0 (Figure 1) was employed to quantify cytokinin dehydrogenase activity in the extracts from the selected transgenic plants. Activities of all seven AtCKXs in tobacco extracts were stable and did not notably decrease within several days after the extraction when stored at 4°C. The highest CKX activities toward iP and iPR substrates were found in leaves of the plants expressing the *AtCKX2* and *AtCKX4* genes. In comparison, the specific activities in plants carrying the *AtCKX1*, *AtCKX3*, *AtCKX5*, *AtCKX6*, or *AtCKX7* gene were significantly lower, elevated not more than 20-fold above the wild-type background values (Figure 2A). This result was consistent within the range of pH from 4.0 to 8.5. On the other hand, AtCKX2- and AtCKX4-expressing plants showed a 1040-fold and 630-fold increase in CKX activity with iP substrate, and 190-fold and 100-fold with iPR as compared to the wild-type plant, respectively. These results show that AtCKX2 and AtCKX4 enzymes are over an order of magnitude more active on iP and its riboside than other AtCKX isoforms under the employed conditions. It is relevant to note that CKX activity in the *AtCKX6* transgenic tobacco was not clearly elevated compared to the wild type, which corresponds well to the subtle phenotypic alterations in these transgenic plants. However, the data obtained by study of the *35S:AtCKX6 Arabidopsis* plants indicated that the gene probably encodes a functional enzyme (Werner and others 2003).

Tobacco plants expressing AtCKX2 and AtCKX4 under the 35S promoter also showed the highest CKX activity *in vivo* when the histochemical localization method utilizing coupled electron acceptors phenazine methosulfate and nitroblue tetrazolium were used (Figure 3A, C, G). Interestingly, the phloem cells were stained much more than other cell types in all tested AtCKX overexpressers, probably because of the smaller cell size of phloem tissue in comparison with the surrounding tissues or a specific transport of CKX protein to the phloem. When the CKX

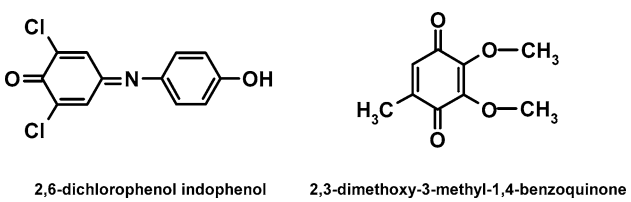


Fig. 1 Structures of electron acceptors of CKX

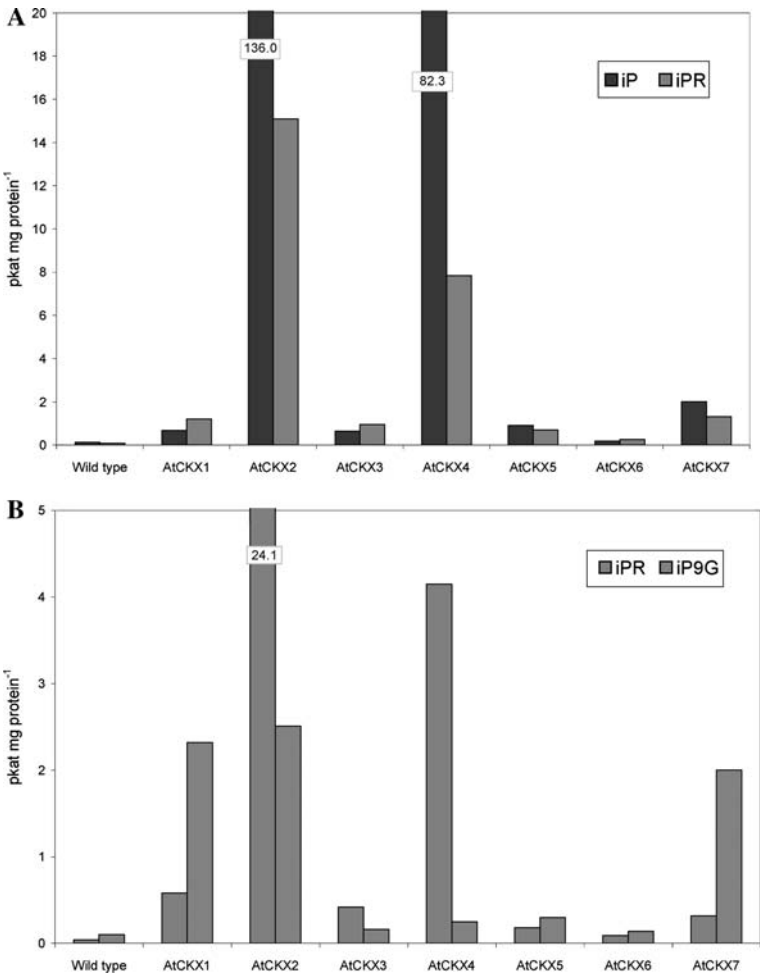


Fig. 2 Cytokinin dehydrogenase activities in a wild-type tobacco and in a crude extract of transgenic tobacco plants overexpressing individual *AtCKX* genes. Activity was determined by the endpoint 4-aminophenol assay (Frébort and others 2002). The reaction mixture contained 5-75 µl of enzyme extract depending on the activity, 0.25 mM substrate (iP, iPR, or iP9G), and 0.5 mM DCPIP (**A**) or Q_0 (**B**) in 0.1 M McIlvaine buffer pH 6.5 (**A**) or pH 5.0 (**B**)

localization experiment was carried out in wild-type maize seedlings, phloem and phloem companion cells also showed the most abundant CKX activity and CKX protein staining (Galuszka and others 2005). Staining of the AtCKX1 in leafstalk phloem cells occurred to a much lower extent than staining of the AtCKX2 (Figure 3E). Under the experimental conditions used in this study, no CKX activity staining was observed with samples of wild-type, AtCKX3, AtCKX5, and AtCKX6 tobacco.

Oxidase vs. Dehydrogenase Activity

A detailed kinetic study on recombinant ZmCKX1 from maize demonstrated that the enzyme, in the presence of a suitable organic electron acceptor, acts preferentially and with high turnover rates in the dehydrogenase mode (Frébortová and others 2004). However, in the absence of an appropriate electron acceptor, ZmCKX1 can utilize molecular oxygen instead and function as an oxidase, though with much lower reaction efficiency (Frébortová and others 2004; Kopečný and others 2005).

To determine the ratio of oxidase to dehydrogenase activity for the AtCKX enzymes, the activity assays were performed with and without artificial electron acceptors and with the iP and iPR substrates present in the reaction mixture at pH 6.5 and pH 8.0 (data not shown). Due to low activity, accurate reaction rates in the presence of oxygen (without electron acceptor) were difficult to quantify for AtCKX5 and AtCKX6 and the ratios could not be determined. Turnover rates of other AtCKX enzymes were significantly lower in the oxidase mode than in the dehydrogenase mode under all tested conditions. For example, the oxidase activities detected for AtCKX1, AtCKX3, and AtCKX7 were approximately 15-40-fold lower than dehydrogenase activities measured in the presence of the electron acceptor DCPIP and the substrate iP, at pH 6.5. For the highly active enzymes AtCKX2 and AtCKX4, the ratios of oxidase/dehydrogenase reaction rates were even lower; the oxidase activities were about 150-300-fold lower than dehydrogenase activities using DCPIP and iP at the same conditions. Although the oxidase activities could be overestimated because of traces of some electron acceptors present in the plant extracts, it is clearly evident

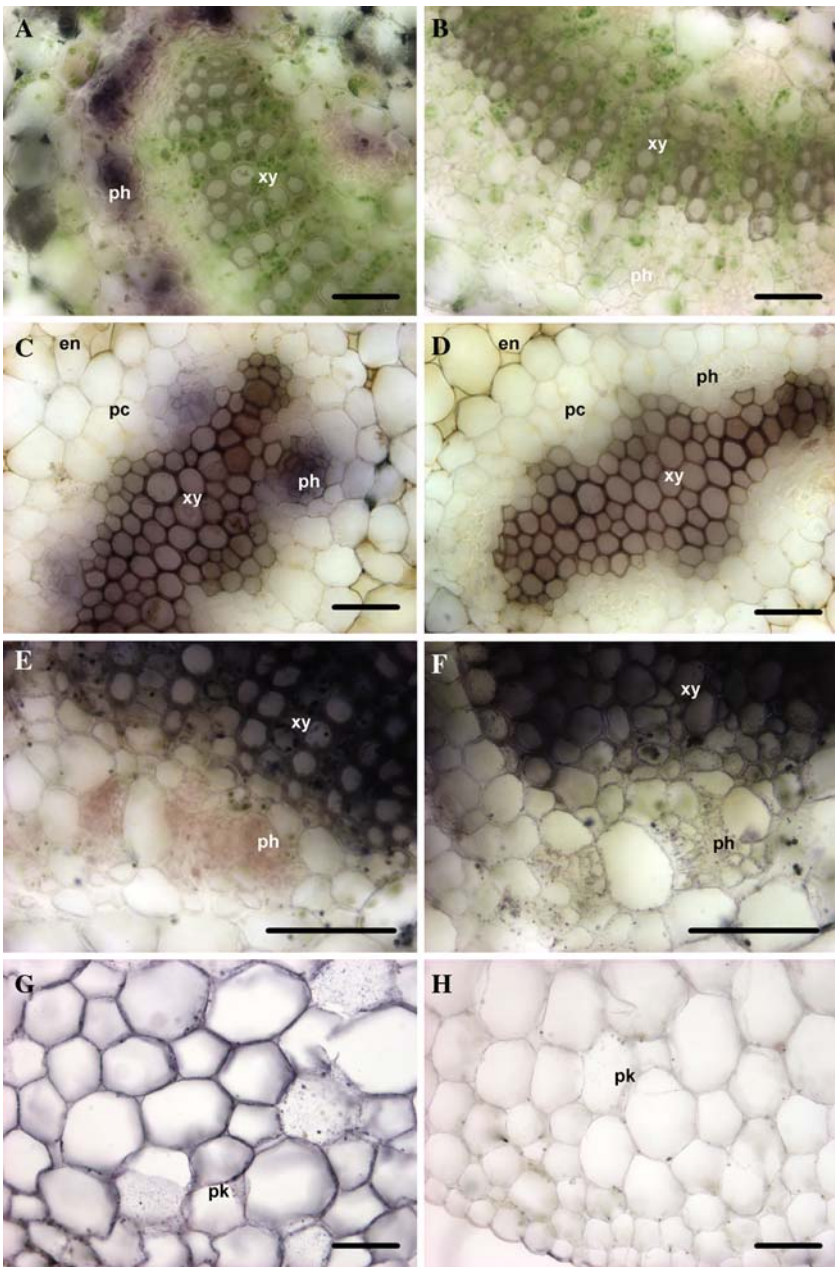


Fig. 3 Histochemical detection of cytokinin dehydrogenase activity with the substrate iP and PMS/NBT staining solution in transgenic tobacco overexpressing the genes *AtCKX1*, *AtCKX2*, and *AtCKX4*. Control experiments were performed with an incomplete staining solution, where the substrate was omitted. Incubation times were 3-14 h, for details see Materials and Methods. *AtCKX2* overexpresser leafstalk, central part (**A**), activity staining (**B**), control experiment, magnification ×200; *AtCKX2* overexpresser root, central part (**C**), activity staining (**D**), control experiment, magnification ×200; *AtCKX1* overexpresser leafstalk, detailed view of vascular tissues (**E**), activity staining (**F**), control experiment, magnification ×400; *AtCKX4* overexpresser leafstalk edge (**G**), activity staining (**H**), control experiment, magnification ×200. Scale bars = 50 µm. en = endodermis, ep = epidermis, pc = pericycle, ph = phloem, pk = parenchyma, xy = xylem

that *in vitro* all AtCKX enzymes function more effectively as dehydrogenases.

Activity with Various Electron Acceptors

Determination of the redox potential of the covalently linked flavin indicated *p*-quinone or a similar compound as the most plausible candidate for a physiologic electron acceptor of ZmCKX1 (Frébortová and others 2004; Galuszka and others 2005). Various electron acceptors with redox potentials and structures different from those of DCPIP (standard redox potential +0.22 V) and Q_0 (+0.08 V) were tested with all AtCKX enzymes. When measured with iP as the substrate, none of the following electron acceptors increased the activity of any of the enzymes more than DCPIP and Q_0: methylene blue (+0.01 V), 1,4-naphthoquinone (+0.07 V), vitamin K_3 (+0.14 V), duroquinone (+0.08 V), 3′,5′-dimethoxy-4′-hydroxyacetophenone, tetrazolium salts (-0.08 V), PMS (+0.08 V), and potassium ferricyanide (+0.44 V). Other compounds such as cytochrome *c* (+0.24 V), vitamin K_1 (+0.13 V), NAD^+ (-0.32 V), $NADP^+$ (-0.32 V), glutathione (-0.23 V), acetyl CoA, syringaldehyde, and salicylic acid were ineffective.

Substrate Specificity within the *Arabidopsis* CKX Family

Figure 4 shows the substrate specificity of all *Arabidopsis* CKX enzymes using electron acceptors Q_0 at pH 5.0 and DCPIP at pH 6.5 and 8.0. All enzymes preferentially cleave isoprenoid cytokinins, but aromatic cytokinins are not resistant to the cleavage. Interestingly, aldehyde products corresponding to the side chains of K, KR, and pT were clearly detected after incubation of these aromatic substrates with AtCKX2 and AtCKX4 enzymes and were distinguishable after prolonged incubation (16 h) with AtCKX1, AtCKX3, and AtCKX7 (data not shown). Although no products were detected after incubation of AtCKX5 and AtCKX6 with K, KR, and pT, one might expect that low reaction rates toward aromatic cytokinins are typical for all CKX enzymes of the *Arabidopsis* family. All AtCKX isoforms are able to effectively degrade both main isoprenoic cytokinins iP and Z and their ribosides (Figure 4). Relative ratios between Z and iP turnovers vary only slightly among the isoforms in the physiologic range of pH; Z was, however, a slightly better substrate than iP.

AtCKX isoforms differ more in the cleavage of glycosylated substrate on the N9 position of the adenine ring (Figures 2B and 4). Although AtCKX1 prefers iPR over iP in the entire pH range, AtCKX2 and AtCKX4 showed a strong preference for free cytokinin bases at neutral and alkaline pH. AtCKX1 affinity for glycosylated cytokinins is enhanced when glucosyl is substituted by ribosyl. AtCKX1 converts iP9G 30-fold more effectively than iP, whereas AtCKX2 at the same pH converts iP9G ten times less efficiently than iP. The same preference for iP9G, notably at acidic pH, was observed for AtCKX7 (see Figure 4). N7-glucosides were not substrates of any of the enzymes (data not shown).

Interestingly, glucosylation on the hydroxyl group of zeatin side chain makes the substrate resistant to cleavage by CKX, whereas O-acetylation does not dramatically reduce the enzymatic cleavage of the substrate side chain. O-acetylzeatin was even found to be the best substrate for AtCKX2 at pH 5.0 (Figure 4).

The ability of CKX to cleave cytokinin nucleotides has never been shown, and spectrophotometric detection of the reaction product 3-methyl-2-butenal by conjugation with 4-aminophenol was a surprising result when iPRMP was incubated as a substrate with AtCKXs. Because partially purified plant extracts were used as a source of the recombinant enzymes, a contribution of tobacco wild-type CKX activity and other cytokinin metabolic enzymes had to be considered. On that account, relative activities obtained with the plant extract containing transgenic AtCKX2 were compared to the activities measured with recombinant AtCKX2 enzyme produced by heterologous expression in *Saccharomyces cerevisiae* and purified to homogeneity (Frébortová and others 2007). No significant differences in substrate specificity and pH preferences between the two enzyme preparations were observed, except the rate of degradation of cytokinin nucleotides. The control experiment with purified recombinant AtCKX2 confirmed, however, that AtCKX2 is indeed able to cleave iPRMP. Although purified AtCKX2 cleaved this substrate at 4% of the rate obtained with iP at pH 6.5, AtCKX2 tobacco extract converted iPRMP under the same conditions at 10% of the rate measured with iP. This result indicates a contribution of endogenous tobacco 5′-mononucleotidase activity to converting cytokinin nucleotides to nucleosides. The contribution of 5′-mononucleotidase activity in other AtCKX tobacco extracts was unfortunately impossible to quantify; thus, the values for iPRMP degradation in Figure 4 may be slightly overestimated. Despite this experimental difficulty, the AtCKX3 enzyme clearly shows preference for the nucleotides as substrates, as evidenced by the high values obtained with iPRMP compared to the values measured with iPR. This high affinity of AtCKX3 for iPRMP was detected under all tested pH conditions. A similar result was observed with AtCKX1 at pH 6.5 and 8.0, which indicates that this enzyme can also convert nucleotides with high turnover rates (Figure 4). Because no specific enzymes that interconvert cytokinin bases to ribosides and nucleotides and vice versa have been found, it is generally thought that these conversions are mediated by the enzymes of purine metabolism. However, conversion of cytokinin ribosides and N-glucosides to free bases by other contaminating enzymes from tobacco extracts is expected to be negligible. Glucosylated cytokinins, with the exception of O- and N^3-glucosides, are not substrates of maize β-glucosidase (Brzobohatý and others 1993). iPR was found to be a weak substrate for wheat adenosine nucleosidase (Chen and Kristopeit 1981), and ZR and iPR were shown to act only as inhibitors for the adenosine nucleosidases extracted from barley (Guranowski and Schneider 1977) and tomato (Burch and Stuchbury 1986).

Activity Assay with Z-type Cytokinins

Data obtained with cytokinin substrates presented in this work showed that the Schiff bases formed by reaction of 4-aminophenol and side-chain aldehydes produced from iP-type and aromatic cytokinins are stable for a period of several hours, whereas the Schiff base of Z-derived side-chain aldehyde, 4-hydroxy-3-methyl-2-butenal, decomposes within 1 h (data not shown). To avoid underestimation of the activities with Z-type substrates, all samples were measured within 5 min after the addition of

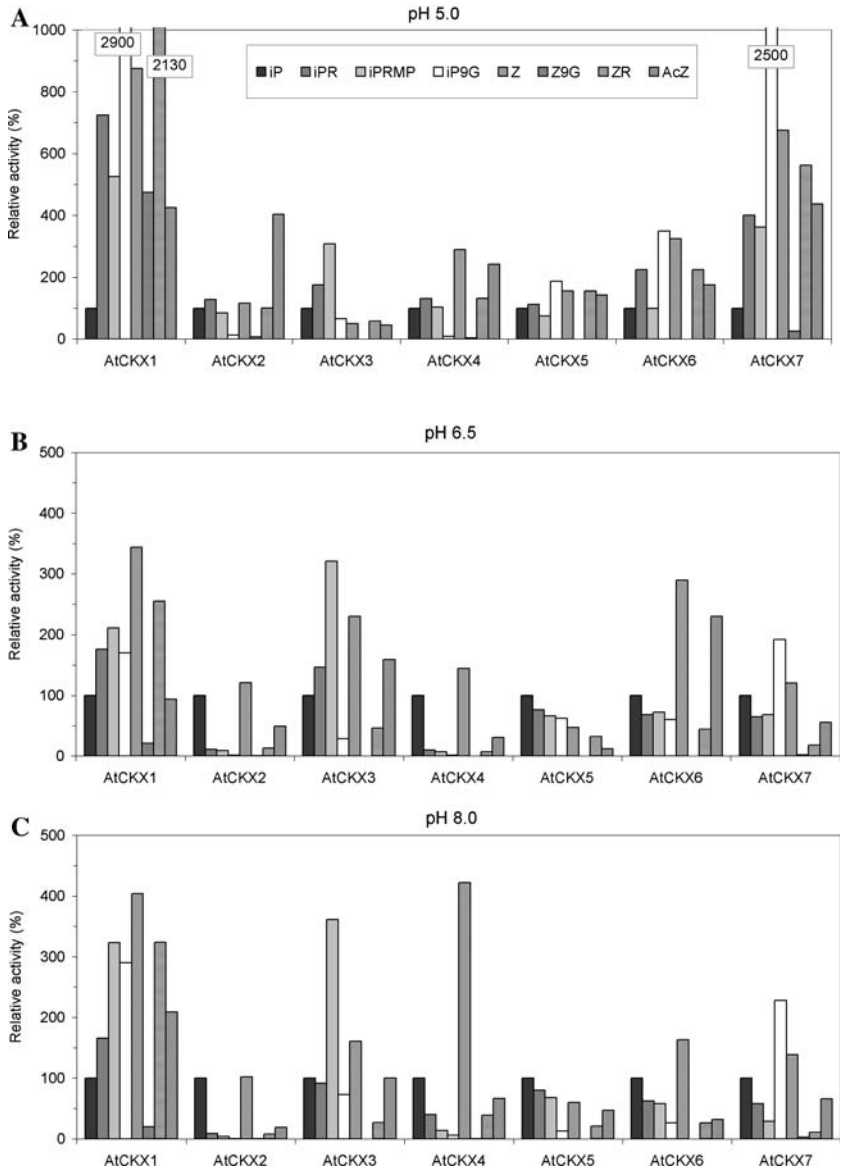


Fig. 4 Substrate specificity of *Arabidopsis* CKX isoforms. The activity with various substrates (0.25 mM) was assayed either in 0.1 M McIlvaine buffer, pH 5.0, with 0.5 mM Q_0 as an electron acceptor (**A**), or in 0.1 M McIlvaine buffer, pH 6.5 (**B**) and pH 8.0 (**C**), with 0.5 mM DCPIP as an electron acceptor. The activity of each isoform with a particular substrate is presented as a percentage of the activity measured with iP (100%). All values represent mean values of data obtained from two parallel extractions; activity toward each substrate was measured in at least three replications. Standard errors for the specific activities were in the range of 5-25%

4-aminophenol reagent. The ratio of iP- and Z-type degradation by the AtCKX2 enzyme was estimated also by the method of DCPIP decolorization (Laskey and others 2003) to exclude possible misinterpretation of data. It was found that a geometric isomer of 4-hydroxy-3-methyl-2-butenal, which arises from cleavage of cisZ, is unable to produce the Schiff base with 4-aminophenol, probably because the *cis*- conformation of the hydroxyaldehyde is more susceptible to self-cyclization than the *trans*- conformation. Although there is no experimental evidence for the chemical behavior of 4-hydroxy-3-methyl-2-butenal, the side chain in the zeatin molecule undergoes self-cyclization in acidic conditions (Haidoune and others 1994). For that reason, degradation of cisZ and other Z-type cytokinin substrates, where the production of Schiff base with 4-aminophenol was not detected after incubation with CKX, was also tested by a continuous method based on DCPIP decolorization. Due to the limitation of this method to determine the activity in crude extracts, only highly active isoenzymes AtCKX2 and AtCKX4 were tested with these substrates. Neither enzyme was able to convert cisZ, DHZ, or ZOG. It is interesting to note that the evolutionarily closest orthologs of AtCKX2 in cereals (ZmCKX1 and HvCKX1 from barley) can process cisZ as a substrate (Bilyeu and others 2001; Galuszka and others 2004). It is possible that the access of cisZ to the catalytic site of CKX is determined by the special structural features of the enzyme active site, and therefore it cannot be excluded that the other AtCKX isoforms may accept cisZ as substrate. This hypothesis is supported by the example of recombinant AtCKX7 protein obtained from yeast that exhibited higher turnovers with cisZ than with Z when measured

using DCPIP decolorization (Bilyeu and Morris, personal communication; our unpublished data). Dihydrozeatin is believed to be resistant to cleavage by CKX, because the reaction product 4-hydroxy-3-methylbutanal was not detected in the reaction mixture, even if an excess amount of maize recombinant enzyme was used (Frébortová and others 2004). To verify if DHZ can be cleaved by AtCKX, the AtCKX isoforms were tested by the 4-aminophenol method. The reaction of CKX with DHZ generally produces 4-hydroxy-3-methylbutanal, but this aldehyde is not commercially available for use as a standard for the control assay. Therefore, its nonhydroxylated analog isovaleraldehyde was used as a standard. A Schiff base produced from 4-aminophenol and isovaleraldehyde can be spectrophotometrically detected with absorption maximum at 315 nm and molar absorption coefficient of 1.1 mM^{-1} cm^{-1}. Incubation of DHZ with any AtCKXs did not give such a product. These results thus corroborate the general presumption that DHZ is completely resistant to cleavage by CKX.

Although data obtained so far indicate that cisZ, DHZ, and ZOG are not cleaved by AtCKX isoforms isolated from tobacco extracts, only future production of purified individual recombinant AtCKXs can conclusively confirm or exclude their reactivity with these and other cytokinins that are less sensitive in colorimetric assays.

pH Optimum of Selected AtCKX Enzymes

To further disclose differences among the AtCKX enzymes, pH optima were determined for four AtCKX isoforms (AtCKX1, AtCKX2, AtCKX4, and AtCKX7) (Figure 5). The activities were screened in the pH range from 4.0 to 8.5 with two substrates, iP and iPR. Because DCPIP is spontaneously reduced in acidic conditions, CKX activities with this electron acceptor were not estimated in the pH range from 4.0 to 5.0. Similarly, Q_0 does not work effectively as an electron acceptor at a pH above 7.5 (Frébort and others 2002).

Using iP as a substrate, the AtCKX enzymes revealed lower activity values with the quinone-type electron acceptor Q_0 than with DCPIP. This result can be explained by the higher redox potential of DCPIP (220 mV) compared to Q_0 (80 mV), which allows faster withdrawal of electrons from reduced FAD, which is the rate-limiting step in the catalytic cycle. Interestingly, degradation of iPR was not significantly affected by the type of electron acceptor used in these experiments. Although AtCKX isoforms tested here mostly revealed sharper activity peaks with iP dependent on pH, especially when DCPIP was used, degradation of iPR by these enzymes was rather pH independent or revealed a plateau over a wider pH range. Figure 5A shows that AtCKX2 protein prefers a neutral and weakly basic pH with Q_0 and DCPIP, respectively, and the pH optimum with iP is around 7.5 (note that the data for iPR are on a scale 10 times lower than data for iP). On the other hand, the AtCKX4 protein prefers weakly acidic and neutral solutions with DCPIP and Q_0, respectively, when iP is used as the substrate (Figure 5B). The AtCKX1 protein exhibits higher specific activities with iPR than with iP and shows the lowest pH optimum from all tested enzymes. When measured with iPR and Q_0, the maximum activity was found at pH 4.5, though the activities around pH 6.0 were similar (Figure 5C). The AtCKX7 enzyme shows a pH optimum around 6.5 with both iP and iPR substrates and the electron acceptor DCPIP (Figure 5D).

Discussion

Transgenic tobacco plants expressing seven *CKX* genes from *Arabidopsis* were tested for cytokinin oxidase/dehydrogenase activity to determine basic biochemical properties of single AtCKX enzymes from the whole family. Biochemical assays showed that only the secreted enzyme AtCKX2 and its closest paralog AtCKX4 displayed high enzymatic activity comparable to that of the well-characterized enzyme from maize ZmCKX1 (Frébortová and others 2004) when tested with isoprenoid cytokinin substrates. Measurements with other AtCKX proteins (AtCKX1, AtCKX3, AtCKX5, AtCKX6, and AtCKX7) showed that they also possessed CKX activity, although their turnover rates were much lower than those of AtCKX2 and AtCKX4. All seven enzymes acted as dehydrogenases rather than as oxidases; they all showed significant increases in activity when measured with an electron acceptor, so none of them was a stringent cytokinin oxidase. The ratios of dehydrogenase/oxidase reactions were higher for AtCKX2 and AtCKX4 (150-300) than for AtCKX1 and other enzymes (15-40) when measured with iP as a substrate at pH 6.5. These variances may be attributed to differences in the electron transfer to the acceptor due to structural diversity of the enzymes. In contrast, no major differences in total activities between less active forms (AtCKX1, AtCKX3) and highly active forms (AtCKX2, AtCKX4) were determined when measured previously in the oxidase mode by a radioisotopic assay (Werner and others 2001). However, these data were probably underestimated in the case of highly active enzymes due to a rapid consumption of the substrate that was present in the reaction mixture at concentrations lower than the K_m value.

Data obtained by this study also confirm that CKX is able to use only low-molecular-weight electron acceptors such as a precursor of ubiquinone Q_0 and artificial dye

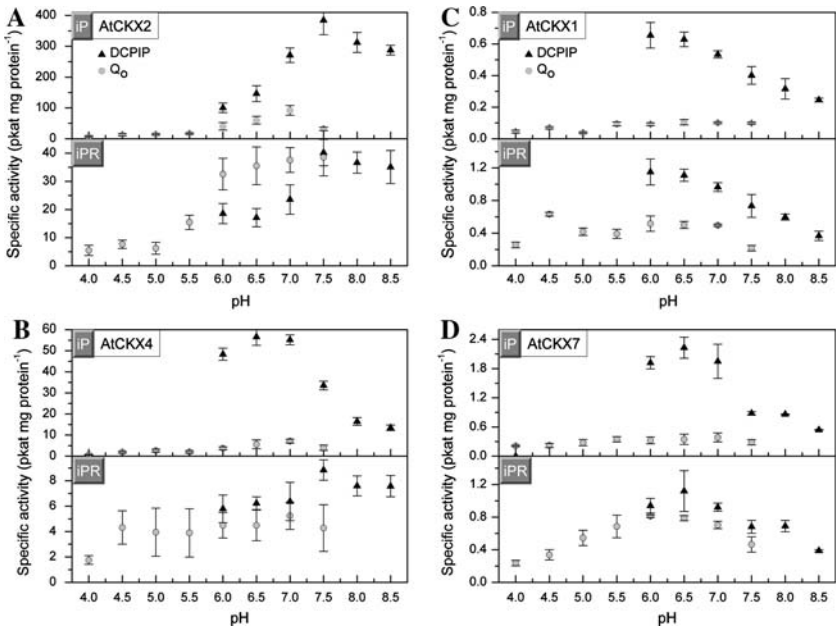
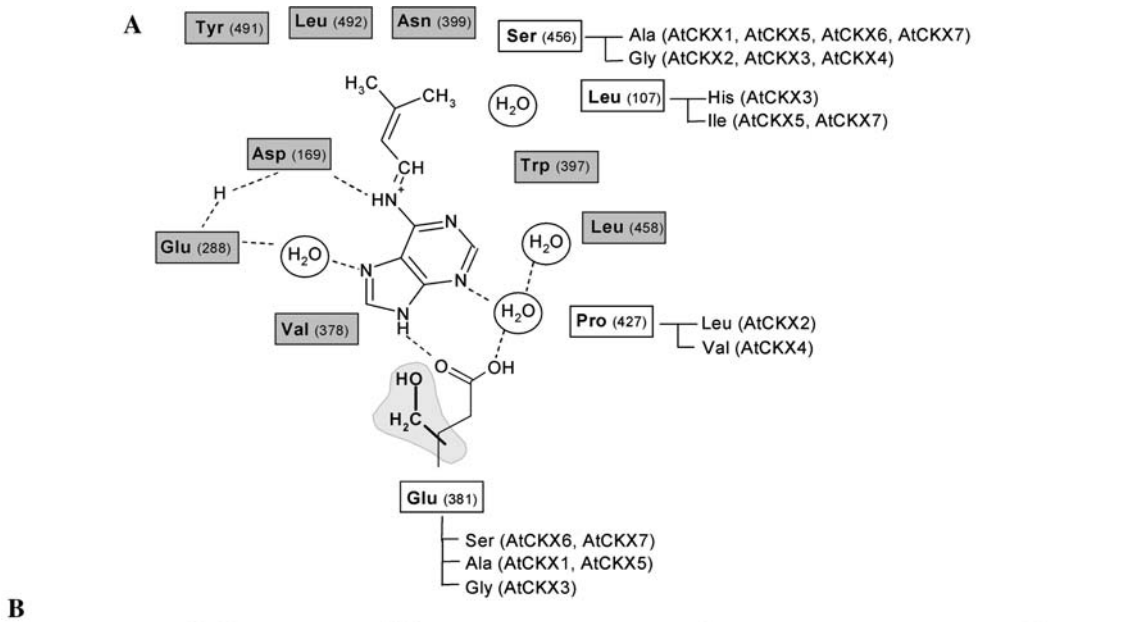


Fig. 5 The pH optima for activities of AtCKX2 (**A**), AtCKX4 (**B**), AtCKX1 (**C**), and AtCKX7 (**D**) determined by 4-aminophenol assay. Reaction mixtures contained 5-75 µl of the enzyme extract, 0.25 mM iP or iPR substrate, and 0.5 mM DCPIP or 0.5 mM Q_0 electron acceptor in McIlvaine buffers of pH from 4.0 to 8.5. Each point represents mean of three to four independent experiments; vertical bars show standard deviation

DCPIP, with a slightly positive standard potential, but not larger molecules such as cytochrome *c* and plantacyanin (Frébortová and others 2004). The presence of a hydrophobic side chain on the molecule of the acceptor seems to be required for dehydrogenation of CKX, since vitamin K_3 (2-methyl-1,4-naphthoquinone) was highly effective but its hydrophobic analog vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) was ineffective. A common feature of all the AtCKX enzymes is that DCPIP gives a higher reaction turnover than the Q_0 acceptor, probably because of its higher redox potential. Our previous work (Frébortová and others 2004) showed that the recombinant ZmCKX1 enzyme also converts isoprenoid cytokinins much more effectively in the presence of electron acceptors such as DCPIP or Q_0 than in the presence of oxygen. It was demonstrated on the maize plants that *in vivo* a suitable electron acceptor for CKX might be generated from phenolic compounds (Galuszka and others 2005). The oxidase turnover rate with iP for the recombinant ZmCKX1 enzyme was determined to be less than one molecule per second. However, in the presence of a suitable electron acceptor it can reach a value of almost one thousand molecules per second (Frébortová and others 2004). The affinity of ZmCKX1 to iP expressed in terms of K_m is almost the same for both oxidase and dehydrogenase reactions, whereas Z, iPR, and ZR showed considerably higher values in the dehydrogenase reaction (Kopečný and others 2005). Nevertheless, the ratio of k_{cat}/K_m, which takes into consideration both affinity and catalytic ability, is significantly higher for the dehydrogenase reaction. The highest ratio of k_{cat}/K_m found for ZmCKX1 and iP reaches 1.3×10^8 M^{-1} s^{-1}, approaching a diffusion limit, in which every collision of the enzyme with the substrate results in catalysis (the enzyme becomes "catalytically perfect"), and the rate of product formation is limited by the diffusion rate and not by the reaction rate. *In planta*, CKX activity is affected by molecular components of the local metabolic resource pool that can serve as electron acceptors directly or after oxidative modification (Galuszka and others 2005). A favorable redox environment in the cell or in the apoplast may allow the enzyme to work at the maximum rate. However, it is still questionable how this enormous catalytic power is used and if it is really needed for the depletion of active cytokinins *in vivo*. In contrast to CKX, cytokinin biosynthetic enzymes isopentenyl transferase AtIPT4 from *Arabidopsis* (Kakimoto 2001) and AIPT from hop (Sakano and others 2004) show lower k_{cat} values by four orders of magnitude. The most active *Arabidopsis* CKX enzymes, AtCKX2 and AtCKX4, degrade cytokinins in the apoplast, where the concentration of cytokinins reflects the balance of accumulation via local synthesis and transport. The rate of cytokinin transport to the apoplast, which can significantly contribute to cytokinin accumulation, is difficult to estimate at the present level of knowledge about plant hormone transportation. Although an equilibrative nucleoside transporter from rice, OsENT2, was recently implicated in the transport of cytokinin ribosides between cytoplasm and apoplast (Hirose and others 2005), it is still not clear how the cytokinins, being predominantly synthesized in plastids, are transported to the cytoplasm (Sakakibara 2006).

Considerable differences in total CKX activities found in the individual AtCKX extracts from tobacco are possibly related to the tertiary structure of the enzymes, including organization of the active site and possibly the level of glycosylation. Enormous differences in specific activity



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ZmCKX1: RDVAYAAFLDRVHGEEVALNKLGLWRVPHPWLNMFVPRSRIADFDRGVFKGILQG-TDIVGPLIVYPLNKSMWDDGMSAATP--SEDVFYAVSLLF---- 459
AtCKX1: SEVPYIEFLDRVHIAERKLRAKGLWEVPHPWLNLLIPKSSIYQFATEVFNNILTS--NNNGPILIYPVNQSKWKKHTSLITP-NE-DIFYLVAFLPSAVP 466
AtCKX2: HDVAYFDFLNRVHVEENKLRSLGLWELPHPWLNLYVPKSRILDFHNGVVKDILLKQKSASGLALLYPTNRNKWDNRMSAMIPEIDEDVIYIIGLLQ---- 429
AtCKX3: KDVTYMDFLNRVRTGELNLKSKGQWDVPHPWLNLFVPKTQISKFDDGVFKGIILRNNITSGPVLVYPMNRNKWNDRMSAAIP--EEDVFYAVGFLR---- 445
AtCKX4: QDVPYFDFLNRVRNEEDKLRSLGLWEVPHPWLNIFVPGSRIQDFHDGVINGLLLNQTSTSGVTLFYPTNRNKWNNRMSTMTP--DEDVFYVIGLLQ---- 450
AtCKX5: TDLQYVDFLDRVHKAELKLRSKNLWEVPHPWLNLFVPKSRISDFDKGVFKGILGN--KTSGPILIYPMNKDKWDERSSAVTP-DE-EVFYLVALLRSALT 447
AtCKX6: TEVAYEAFLDRVHVSEVKLRSKGQWEVPHPWLNLLVPRSKINEFARGVFGNILTD--TSNGPVIVYPVNKSKWDNQTSAVTP-EE-EVFYLVAILTSASP 424
AtCKX7: VDLPYVDFLLRVKRSEEIAKENGTWETPHPWLNLFVSKRDIGDFNRTVFKELVKN--GVNGPMLVYPLLRSRWDDRTSVVIP-EEGEIFYIVALLR-FVP 447
            *  ** **  *       *  ******      *  *  *           *    **    *   *  *       *   *

ZmCKX1: SSVAPNDLARLQEQNRRILRFCDLAGIQYKTYLARHTDRSDWVRHFGAAKWNRFVEMKNKYDPKRLLSPGQDIFN------------------------ 534
AtCKX1: NSSGKNDLEYLLKQNQRVMNFCAAANLNVKQYLPHYETQKEWKSHFG-KRWETFAQRKQAYDPLAILAPGQRIFQKTTGKLSPIQLAKSKATGSPQRYHY 565
AtCKX2: SAT-PKDLPEVESVNEKIIRFCKDSGIKIKQYLMHYTSKEDWIEHFG-SKWDDFSKRKDLFDPKKLLSPGQDIF------------------------ 501
AtCKX3: SAG-FDNWEAFDQENMEILKFCEDANMGVIQYLPYHSSQEGWVRHFG-PRWNIFVERKYKYDPKMILSPGQNIFQKINSS-------------------- 523
AtCKX4: SAGGSQNWQELENLNDKVIQFCENSGIKIKEYLMHYTRKEDWVKHFG-PKWDDFLRKKIMFDPKRLLSPGQDIFN------------------------ 524
AtCKX5: DGEETQKLEYLKDQNRRILEFCEQAKINVKQYLPHHATQEEWVAHFG-DKWDRFRSLKAEFDPRHILATGQRIFQNPSLSLFPPSSSSSSAASW------ 540
AtCKX6: GSAGKDGVEEILRRNRRILEFSEEAGIGLKQYLPHYTTREEWRSHFG-DKWGEFVRRKSRYDPLAILAPGHRIFQKAVSYS------------------- 504
AtCKX7: PCAKVSSVEKMVAQNQEIVHWCVKNGIDYKLYLPHYKSQEEWIRHFG-NRWSRFVDRKAMFDPMAILSPGQKIFNRSL---------------------- 524
                  *                  **       *  ***   *  *   *   **   *  *  **
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Fig. 6 Schematic representation of cytokinin binding in the CKX enzyme. **A** Oxidized isopentenyladenine bound in ZmCKX1 (PDB entry 1W1Q) is surrounded by amino acid residues that are highly conserved among the members of the *Arabidopsis* CKX family. Fully conserved residues are shown in gray boxes, amino acid alterations toward the structure of ZmCKX1 are depicted (numbering and positioning according to Malito and others 2004). Serine (shaded residue) that replaces Glu381 in the structure of AtCKX7 (PDB entry 2EXR) was positioned in the scheme on the basis of structural alignment done by PyMOL and may also form a hydrogen bond to N9 of the adenine ring. This structural residue, replaced by Ala or Gly in other *Arabidopsis* enzymes, could play a crucial role in determining substrate specificity toward N9-substituted cytokinins. **B** ClustalW alignment (BioEdit) showing distribution of the cytokinin-binding residues in the protein sequence; asterisks show amino acid identity, and the arrow shows the cytokinin N9 hydrogen-bonding residue (Glu381 in ZmCKX1)

were also observed between recombinant AtCKX1 and AtCKX2 enzymes when expressed in yeast (Frébortová and others 2007). Phylogenetic analysis based on the amino acid sequence alignment of the AtCKX protein family shows that AtCKX2 and AtCKX4 are the proteins most similar to ZmCKX1, whereas other members of the *Arabidopsis* family display higher diversity (Popelková and others 2004). The hypothesis presented here, that differences in the substrate specificity of AtCKXs may be closely related to small structural variations in the vicinity of the enzyme active sites, comes from the fact that a Glu residue (Glu381), which was shown in crystals of an iP-ZmCKX1 complex to form a hydrogen bond with the N9 atom of the adenine ring of iP (Malito and others 2004), is conserved only in the amino acid sequence of the highly active *Arabidopsis* enzymes AtCKX2 (Glu348) and AtCKX4 (Glu371). The less active enzymes, AtCKX1, AtCKX3, AtCKX5, AtCKX6, and AtCKX7, contain different amino acids in this position (Figure 6A). Structural alignment of the crystal structures of ZmCKX1 (Malito and others 2004) and AtCKX7 (Wesenberg and others 2005) and an amino acid sequence alignment of ZmCKX1 with other AtCKX proteins revealed that the shape and amino acid composition of the active site are highly conserved with respect to the substrate surrounding residues (Figure 6B). A subtle variation in this conserved set of amino acids is likely to contribute significantly to the substrate binding and turnover rates of the individual substrates. In

theory, the substitutions of the Glu348 or Glu371 residue with hydrophobic ones in AtCKX1, AtCKX3, and AtCKX5 or with Ser in AtCKX6 and AtCKX7 can cause less tight binding of the free cytokinin base, which would be in good agreement with the lower activity of these enzymes with iP. In the case of iPR, whose affinity is generally lower and the differences in activities of the individual AtCKX enzymes are less distinct (Figure 2), the hydrogen bonding of Glu to the N9 atom of adenine may not be established due to the protruding ribosyl moiety.

In summary, the results of this study show that the highly active secreted enzymes AtCKX2 and AtCKX4 prefer free cytokinin bases as substrates at neutral and slightly basic pH. Along with the other presumably secreted enzymes AtCKX5 and AtCKX6 (Werner and others 2003), they may regulate the level of cytokinins in the apoplast. However, the pH optima of these enzymes with iP do not reflect the fact that pH in the apoplast is generally around 5.0. AtCKX1 shows the highest activity at weakly acidic pH, with extensive preference for cytokinin ribosides and N^9-glucosides, which is in accordance with the localization of this enzyme in vacuoles (Werner and others 2003) having an average pH value around 5.5. In higher plants, secondary metabolites are often converted to glycoconjugates, which then accumulate in vacuoles. Glucosides of cytokinins are nonactive forms that are probably also stored in vacuoles (Fusseder and Ziegler 1988). Thus, AtCKX1 might act there as a factor that recycles components of cytokinin molecules. Similarly, AtCKX7, also with a high preference for N^9-glucosides, may perform this function in the cytoplasm. The physiologic meaning for such a preference is unclear so far because N^9-glucosides were found to be inactive in many biotests (Letham and others 1983; Spíchal and others 2004), and thus a requirement for their rapid degradation via CKX seems to be irrelevant *in vivo*. Finally, the AtCKX3 protein is the isoform that is likely to preferentially degrade cytokinin nucleotides. Neither AtCKX enzyme exhibited high activity with aromatic cytokinins; however, naturally occurring aromatic cytokinins such as hydroxy derivatives of benzyladenine could also be catabolized at low rates *in vivo* as it was first shown with partially purified wheat CKX (Laloue and Fox 1989).

The correlation between biochemical characteristics of the individual AtCKX proteins and their molecular structure requires further research, including cloning, expression, and purification of the recombinant enzymes followed by crystallographic and spectroscopic analyses. Crystallization of AtCKX7 (Wesenberg and others 2005) and cloning, expression, and purification of AtCKX2 from *Saccharomyces cerevisiae* (Werner and others 2001; Frébortová and others 2007) are the first promising steps in this direction.

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