Vacuolar and cytosolic cytokinin dehydrogenases of Arabidopsis thaliana: Heterologous expression, purification and properties

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1. Introduction

Cytokinins are important signaling molecules regulating cell division and differentiation in plant cells (Mok and Mok, 2001). Not all forms of cytokinins are biologically active and it is likely that the concentrations of both active and inactive cytokinins are actively managed. Concentrations of active cytokinins and their distribution in plant tissues are controlled by irreversible degradation (Hare and van Staden, 1994; Galuszka et al., 2000) as well as through synthesis and compartmentation. The enzyme playing a key role in cytokinin catabolism is cytokinin dehydrogenase (CKX; EC 1.5.99.12), a flavoprotein that cleaves off the N6-side chains from cytokinins is a flavoprotein classified as cytokinin dehydrogenase (CKX; EC 1.5.99.12), CKXs also show low cytokinin oxidase activity, but molecular oxygen is a comparatively poor electron acceptor. The CKX gene family of Arabidopsis thaliana comprises seven members. Four code for proteins secreted to the apoplastic, the remainder are not secreted. Two are targeted to the vacuoles and one is restricted to the cytosol. This study presents the purification and characterization of each of these non-secreted CKX enzymes and substrate specificities are discussed with respect to their compartmentation. Vacuolar enzymes AtCKX1 and AtCKX3 were produced in Pichia pastoris and cytosolic enzyme AtCKX7 was expressed in Escherichia coli. The recombinant proteins were purified by column chromatography. All enzymes preferred synthetic electron acceptors over oxygen, namely potassium ferricyanide and 2,3-dimetoxy-5-methyl-1,4-benzoquinone (Q0). In slightly acidic conditions (pH 5.0), N6-(2-isopen-tenyl)adenine 9-glucoside (iP9G) was the best substrate for AtCKX1 and AtCKX7, whereas AtCKX3 preferentially degraded N6-(2-isopen-tenyl)adenine 9-riboside-5'-monophosphate (iPMP). Moreover, vacuolar AtCKX enzymes in certain conditions degraded N6-(2-isopentenyl)adenine di- and triphosphates two to five times more effectively than its monophosphate.
FAD-binding and substrate-binding domains, but the sequences outside these domains display strong divergence (Popelková et al., 2004). AtCKX enzymes differ in their biochemical properties, in regulation of their expression and in subcellular localization (Schmülling et al., 2003). N-terminal sequences of AtCKX proteins indicate that four of them (AtCKX2, AtCKX4, AtCKX5 and AtCKX6) are targeted to the plant secretory pathway, whereas AtCKX7 is probably localized to the cytosol due to the lack of any recognized signal peptide. Finally AtCKX1 and AtCKX3 were initially predicted to be imported into mitochondria (TargetP and PSORT programs, Emanuelsen et al. (2000) and Nakai and Horton (1999), respectively), but the study of their GFP fusion proteins confirmed their presence in vacuoles (Werner et al., 2003).

Purification of CKX enzymes from natural sources like maize (Morris et al., 1999), wheat or barley (Galuszka et al., 2001) posed an extreme challenge and required several advanced purification steps. In general CKX proteins show low retention on chromatographic columns and are difficult to concentrate by salting out. Fortunately they possess very stable activity. Recombinant CKX enzymes from A. thaliana were individually expressed in transgenic tobacco plants (Werner et al., 2001) and the plant extracts examined for CKX activity and substrate specificity (Galuszka et al., 2007). Heterologous expression of AtCKX2, the most abundant secreted Arabidopsis CKX enzyme, has been achieved in Saccharomyces cerevisiae (Werner et al., 2001; Frébortová et al., 2007) and Pichia pastoris (Bilyeu et al., 2001) allowing purification and biochemical characterization (Frébortová et al., 2007).

Non-secreted AtCKX enzymes have not previously been studied thoroughly. The crystal structure of cytosolic AtCKX7 protein has been reported (Bae et al., 2008) as an output from an eukaryotic structural genomics pipeline, but its activity or catalytic properties were not investigated. In this work, recombinant AtCKX was obtained from Escherichia coli BL21 (DE3) STAR lysate and vacuolar proteins AtCKX1 and AtCKX3 were purified from P. pastoris, using directed secretion to the culture medium. We determined the properties of all three enzymes purified to homogeneity.

2. Results and discussion
2.1. Targeting of AtCKX proteins to vacuoles
AtCKX proteins have different subcellular localizations and earlier work has shown that AtCKX1 (GenBank accession no. NP_181682) and AtCKX3 (NP_205007) are vacuolar (Werner et al., 2003) by means of GFP fusions in transgenic A. thaliana plants. It was supposed that vacuolar targeting might be specific to certain cell types since AtCKX3 was found mainly in central vacuoles, whereas AtCKX1 only in smaller vacuoles (Werner et al., 2003). As part of the P. pastoris expression strategy, the sequence of AtCKX3 was examined for targeting motifs (SignalP 3.0 Server, Bendtsen et al., 2004). Initial expression of AtCKX3, however, was not efficient and no activity was detected in the transformed yeast culture. Further sequence analysis of AtCKX3 revealed the presence of a sequence similar to an N-terminal sequence-specific vacuolar sorting signal (ssVSS). This motif targets proteins to lytic vacuoles; it is typically present in the protein after the N-terminal signal peptide. Finally AtCKX3 recombinant protein with a yeast signal peptide and the vacuolar sorting signal deleted was properly processed by the yeast and secreted to the medium in its active form. The NPIR consensus (Nakamura and Matsuoka, 1993; Vitale and Raikhel, 1999). When a re-designed gene construct was prepared, AtCKX3 recombinant protein with a yeast signal peptide and the vacuolar sorting signal deleted was properly processed by the yeast and secreted to the medium in its active form. The NPIR consensus was also found in the sequence of AtCKX1 (Fig. 1) and deleted before the expression in Pichia. The activity of the NPIR motif in yeast complements the earlier GFP data to confirm that these CKX isoforms are directed to vacuoles. Deletion of the motif was essential for satisfactory expression in yeast.

2.2. Selection of vectors and expression system
Vacular AtCKX enzymes were first expressed in pGAPZα vector to confirm the protein secretion to Pichia growth medium by CKX activity assay. In order to facilitate purification, recombinant proteins were fused with a C-terminal His-tag domain. Initial experiments showed that C-terminal His-tag fusion (in pGAPZα) decreases CKX specific activity (67% activity loss), therefore a novel vector was prepared from pGAPZαA by inserting a coding sequence for 10× His between the α-factor coding sequence and the multiple cloning site. AtCKX genes were subsequently cloned into pGAPZαA[His]10 and expressed in P. pastoris X-33. Expression of heterologous proteins in fermentor cultures can result in accumulation of vacuolar proteases such as proteinase A (pep4) with resulting damage to the protein of interest (Lin-Cereghino and Lin-Cereghino, 2007). For that reason an auxotrophic and protease-deficient strain SMD1168 (his4, pep4) was chosen and new vector constructs were prepared. The expression cassette of pGAPZαA[His]10:AtCKX containing constitutive GAP (glyceraldehyde 3-phosphate dehydrogenase) promoter and AtCKX gene was inserted into HIS4-based vector pPIC9K (Supplementary Fig. 1). The resulting vectors gave acceptable expression levels of vacuolar AtCKX proteins and were selected for large scale expression in a fermentor.

Cytosolic enzyme AtCKX7 was expressed intracellularly in P. pastoris in a shake flask but the protein purification from lysate was very difficult and insufficient (data not shown). Therefore expression in E. coli was chosen with the vector pTYB12 that introduces a chitin-binding intein tag to enable fast and easy purification.

2.3. Production of recombinant AtCKX proteins
AtCKX1 and AtCKX3 genes were expressed in P. pastoris and active proteins were released into the growth medium. Screening for activity used assay conditions described earlier (Galuszka et al., 2007). Yeasts were grown first in 250 ml Erlenmeyer flasks to confirm CKX activity and then the process was scaled up using a fermentor. After fermentation, approximately 8 l of culture media were concentrated by ultrafiltration and purified by column chromatography (Table 1). Ammonium sulfate precipitation was ineffective, even at 100% saturation, probably due to the presence of a silicone antifoam agent that was added in the fermentor. First chromatographic step (Octyl Sepharose or hydroxyapatite for AtCKX1 and AtCKX3, respectively) resulted in low protein recovery since much CKX was washed out from the column together with the antifoam agent during an isocratic wash at the start of the chromatographic run. Ni affinity chromatography was ineffective at early stage and later on gave only moderate purification. 95% pure AtCKX1 was eluted from a Hema-Bio Phenyl column and AtCKX3 was purified on Resource Q column.

In general, purification of vaccular AtCKX proteins was problematic. The use of the silicone antifoam agent was essential in the fermentor, but compromised column chromatography. The proteins also exhibited low polarity. Overall yields were low (1.3% and 0.5% for AtCKX1 and AtCKX3, respectively), but acceptable. Similar difficulties caused by the low polarity of CKX were previously described for recombinant AtCKX2 (Frébortová et al., 2007) and for enzymes from wheat and barley (Galuszka et al., 2001). It was not the case for recombinant ZmCKX1 (Bilyeu et al., 2001; Kopecˇny´ et al., 2005) therefore it is suspected that low polarity of CKX may be related to the amino acid sequence, the structure
of the protein and post-translational modifications (Frébortová et al., 2007).

Cytosolic AtCKX7 protein was expressed in *E. coli* with IPTG induction. The bacterial cells were disrupted using a French press and the enzyme was purified from lysate (50-fold, 33% recovery) in a single step on a chitin resin column, giving a protein with an activity of 181 nkat/mg with 250 \( \mu \)M iP in pH 7.0. In 2008, the crystal structure of AtCKX7 was reported (without measuring any data on enzymatic activity); the protein was prepared by the expression in CESG vector pVP13-GW, where the protein was bearing N-terminal (His)_6-MEB fusion tag enabling a generic Ni-IMAC purification strategy. The 33 N-terminal residues were not assigned in the model (Bae et al., 2008).

The purity of each AtCKX protein was examined by SDS–PAGE (Fig. 2). AtCKX1 and AtCKX3 proteins migrated as single sharp bands corresponding to molecular mass of approximately 97 and 92 kDa, respectively, whereas AtCKX7 gave one sharp band of molecular mass of about 60 kDa and a weaker band of 120 kDa. Using an immunoblot analysis (Fig. 2), each of the above bands gave positive staining with a polyclonal antibody raised against barley HvCKX2 (Galuszka et al., unpublished results), suggesting that in the case of AtCKX7 the upper band may be an aggregated form of the protein (a dimer). Anomalously high sizes of vacuolar proteins seen on the SDS–PAGE gel were most certainly caused by the used method (tricine–SDS–PAGE). Theoretical molecular masses for AtCKX1 and AtCKX3 are 65.05 and 59.72 kDa, respectively. This was proved by SDS–PAGE according to Laemmli (1970) as well as by MALDI-TOF intact protein mass measurement (64290.1 Da for AtCKX1, 58080.4 Da for AtCKX3 and 57057.3 Da for AtCKX7).

The identity of each of the recombinant and purified enzymes was confirmed using MALDI-TOF peptide mass fingerprinting after in-gel digestion (Supplementary Fig. 2). Recombinant AtCKX1 was unambiguously assigned to *A. thaliana* cytokinin oxidase/dehydrogenase 1 in the NCBInr database (accession no. gi|15227374; 19 matched peptides; MOWSE score: 120; sequence coverage: 30%). Recombinant AtCKX7 was unambiguously assigned to *A. thaliana* cytokinin oxidase/dehydrogenase 7 in the NCBInr database (accession no. gi|30688201; 22 matched peptides; MOWSE score: 224; sequence coverage: 46%). Recombinant AtCKX3 was unambiguously assigned to *A. thaliana* cytokinin oxidase/dehydrogenase 3 in the NCBInr database (accession no. gi|15241997; 17 matched peptides; MOWSE score: 146; sequence coverage: 35%). For clarity, tryptic peptides with \( m/z \) 943 and 1437 were subjected to a MALDI post-source decay analysis, which yielded sequences WNIFVER and FYIDFSEFTR, respectively, upon reading b- and y-ion fragment series, both in accordance with the primary structure of AtCKX3.

### Table 1

Purification of recombinant vacuolar AtCKXs. Activities were determined for 250 \( \mu \)M iP, in McIlvaine buffer of pH 5.0 and with 500 \( \mu \)M Qp.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein content (mg/ml)</th>
<th>Specific activity (pkat/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtCKX1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
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<td>0.03</td>
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<td>1.00</td>
<td>100</td>
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<td>47</td>
<td>1.2</td>
<td>95.7</td>
</tr>
<tr>
<td>Octyl Sepharose</td>
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<td>1.6</td>
<td>20.2</td>
</tr>
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<td>11</td>
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<tr>
<td>Ni-Sepharose</td>
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<td>0.96</td>
<td>539</td>
<td>14</td>
<td>7.7</td>
</tr>
<tr>
<td>HEMA-BIO Phenyl</td>
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<td>0.096</td>
<td>1583</td>
<td>41.7</td>
<td>1.3</td>
</tr>
<tr>
<td>AtCKX3</td>
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<td></td>
<td></td>
<td></td>
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<td>Crude extract</td>
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<td>0.05</td>
<td>89</td>
<td>1.00</td>
<td>100.0</td>
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<tr>
<td>Ultrafiltration</td>
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<td>57.9</td>
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<tr>
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<td>2.39</td>
<td>18.3</td>
</tr>
<tr>
<td>Ni-Sepharose</td>
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<td>12.1</td>
<td>218</td>
<td>2.45</td>
<td>8.1</td>
</tr>
<tr>
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<td>0.46</td>
<td>1607</td>
<td>18.01</td>
<td>0.5</td>
</tr>
</tbody>
</table>

### Fig. 1

Identification of the vacuolar targeting sequence in AtCKX1 and AtCKX3. N-terminal sequence-specific vacuolar sorting signal (ssVSS) that typically it contains degenerate signal (N/L)–(P/I)L–(I/P)–R/N/S called also NPR consensus was found. The ssVSS is underlined by a dotted line and the arrows indicate the predicted end of a signal peptide sequence (SignalP 3.0 Server; thin arrows) and a peptide sequence that had to be cleaved to achieve secretion of active proteins from the yeast cells (thick arrows), respectively.

### Fig. 2

SDS–PAGE and immunoblotting of non-secreted AtCKX proteins. After the purification, AtCKX proteins (6 \( \mu \)g each) were subjected to tricine–SDS–PAGE (8% polyacrylamide gel) and either stained by Coomassie Brilliant Blue (lanes 1–3) or electroblotted onto PVDF membrane and detected using the polyclonal antibody against the C-terminal fragment of barley HvCKX2 by chemiluminescent visualization (lanes 5–7). Lane description: 1 and 7 – AtCKX7, 2 and 6 – AtCKX3, 3 and 5 – AtCKX1, and 4 – molecular mass marker.
2.4. Properties of non-secreted AtCKX enzymes

It is likely that each cellular compartment will contain different cytokinins and different cytokinin conjugates and so there is an interest to investigate how substrate specificities vary between each of the CKX isoforms. Consequently, purified recombinant enzymes were characterized for their preference in both electron acceptor and substrate using a modified end-point method (Frébort et al., 2002). Specific activities of each enzyme were measured with iPR as a substrate at both pH 5.0 and pH 7.5 using a set of electron acceptors. The basal level of enzyme activity using molecular oxygen as an acceptor was used as a reference (Galuszka et al., 2001; Frébortová et al., 2004).

In slightly acidic conditions, ferricyanide was found to be the preferred electron acceptor for all three enzymes, enhancing enzymatic activity 552-fold, 7.2-fold and 59.5-fold for AtCKX1, AtCKX3 and AtCKX7, respectively (Table 2). At neutral pH, AtCKX7 preferred DCPIP as the electron acceptor (15.4-fold higher activity), whereas vacuolar AtCKX enzymes gave higher reaction rates with Q0 (238-fold for AtCKX1 and 2.7-fold for AtCKX3, respectively). It is noticeable that the activity of AtCKX3 was only modestly enhanced over the activity with oxygen, suggesting that this enzyme is either a less effective dehydrogenase compared to other AtCKXs, or prefers a different, but unknown electron acceptor.

Having established that all enzymes performed well with Q0 as electron acceptor, substrate specificities were assessed in 500 μM Q0. For ease of comparison, data are presented relative to the specific activity with iP as substrate. Both AtCKX1 and AtCKX7 showed considerable preference for certain conjugates over iP. Interestingly, iP9G was the best substrate for AtCKX7 and one of the best substrates for AtCKX1 (approx 40-fold increase in activity above iP in each case), although being a poor substrate for AtCKX3 (Fig. 3). AtCKX1 showed highest preference for Tz phosphates (up to 96-fold elevation of activity for tZTP over iP) and also effectively degraded iP phosphates (up to 30-fold for iPTP). Surprisingly, AtCKX3 did not demonstrate strong preference for any studied cytokinin, even though preferred iP phosphates and iP over iP (less than 3-fold).

AtCKX7 also very efficiently degraded Tz and iPR (20-fold and 4.5-fold, respectively). Similar preference for Tz as well as for iP9G was previously observed for cytosolic ZmCKX10 (Šmehilová et al., 2009), while apoplastic ZmCKX1 and AtCKX2 degrade Tz most efficiently and have very low activity with iP9G and iPMP (Bilyeu et al., 2001; Šmehilová et al., 2009; Frébortová et al., 2007; Galuszka et al., 2007). None of the intracellular AtCKX enzymes showed high activity with aromatic cytokinins. AtCKX7 was able to degrade kinetin at 28% of the rate for iP and AtCKX1 was able to degrade kinetin riboside at 50% of the rate for iP. No activity was recorded against kinetin or its riboside for AtCKX3.

Activities using oxygen as the electron acceptor were generally much lower (Table 2), but substrate preferences were essentially unchanged (Fig. 3b).

Reaction of vacuolar and cytoplasmic AtCKX enzymes with cytokinin nucleotides was further studied by incubating the purified enzymes in a mixture of nine substrates and analyzing residual cytokinins by capillary electrophoresis (Fig. 4). In general, iP phosphates were better substrates than zeatin phosphates for all AtCKX enzymes studied (cis- and trans-zeatin phosphates were

Table 2
CKX specificity for the electron acceptor. Recombinant non-secreted AtCKX proteins were compared in their CKX activities in the presence of 500 μM electron acceptor in McIlvaine buffer pH 5.0 and 7.5 with 250 μM iPR as a substrate. The values show ratio of CKX activities to the activity obtained without externally added electron acceptor (with O2).

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>AtCKX1 pH 5.0</th>
<th>AtCKX3 pH 5.0</th>
<th>AtCKX7 pH 5.0</th>
<th>AtCKX1 pH 7.5</th>
<th>AtCKX3 pH 7.5</th>
<th>AtCKX7 pH 7.5</th>
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<tbody>
<tr>
<td>Oxygen</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3’,5’-Dimethoxy-4-hydroxyacetophenone</td>
<td>0.7</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.8</td>
<td>0.3</td>
<td>1.9</td>
<td>0.9</td>
<td>1.5</td>
<td>2.1</td>
</tr>
<tr>
<td>2,3,5-Triphenyl-tetrazolium chloride</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0.9</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>552</td>
<td>130</td>
<td>7.2</td>
<td>1.3</td>
<td>59.5</td>
<td>2.9</td>
</tr>
<tr>
<td>NAD</td>
<td>1.5</td>
<td>1.4</td>
<td>0.8</td>
<td>0.8</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>2,6-Dichlorophenol indophenol</td>
<td>25</td>
<td>49</td>
<td>0.6</td>
<td>0.2</td>
<td>2.6</td>
<td>15.4</td>
</tr>
<tr>
<td>2,3-Dimetoxy-5-methyl-1,4-benzoquinone (Q0)</td>
<td>117</td>
<td>238</td>
<td>3.2</td>
<td>2.7</td>
<td>19.9</td>
<td>5.6</td>
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</table>

Fig. 3. Comparison of substrate specificities of non-secreted AtCKX proteins given as relative reaction rates towards iP (100%). Measurements were done in McIlvaine buffer (pH 5.5) with 200 μM substrates and either 500 μM electron acceptor Q0 (2,3-dimetoxy-5-methyl-1,4-benzoquinone) for dehydrogenase reaction (A) or no electron acceptor added for oxidase reaction (B). Specific activity with iP was 266 pkat/mg for AtCKX1, 416 pkat/mg for AtCKX3 and 1776 pkat/mg for AtCKX7.
impossible to separate due to their migration as one peak). Vacuolar AtCKX enzymes preferentially degraded tri- and then diphosphates over monophosphates of isopentenyladenine. Insignificant degradation of zeatin monophosphates by either AtCKX1 or AtCKX3 was observed after the 3-h incubation, in contrast to tri- and diphosphates which rapidly disappeared from the mix, especially in the case of AtCKX1 (Fig. 4a and b). The cytosolic enzyme AtCKX7 preferred zeatin monophosphate over di- and triphosphates, however these activities were trivial compared to those for iP phosphates (Fig. 4c). Under the same conditions, relative reaction rates for AtCKX1, AtCKX3 and AtCKX7 against iPMP vs. iP were 13, 10 and 1, respectively. In contrast, the secreted enzymes AtCKX2 and ZmCKX1 had reaction rates for iPMP lower by two orders of magnitude than for iP and the degradation of diphosphates was even less favoured, close to the detection limit of the assay method (data not shown).

3. Concluding remarks

Amino acid sequence analysis of both AtCKX1 and CKX3 revealed that they contain degenerate N-terminal sequence-specific vacuolar sorting signal (ssVSS) consisting of four amino acids known as the NPIR consensus. For the first time recombinant vacuolar and cytoplasmic cytokinin dehydrogenase enzymes from A. thaliana were obtained and characterized. Their preferences towards different electron acceptors and preferences towards different cytokinins and cytokinin nucleotides were determined. It was found that the activity of vacuolar AtCKX1 protein strongly depends on the presence of an appropriate electron acceptor. Vacuolar AtCKX3 and cytosolic AtCKX7 showed less preference for electron acceptor, but remained more efficient as dehydrogenases than as oxidases (Table 2). In contrast to the substrate specificities recorded for secreted CKXs, all three non-secreted AtCKX proteins were able to cleave all types of endogenous cytokinin nucleotides. It is interesting to consider on the preference of AtCKX1 and AtCKX7 isoforms for cytokinin N-glucoside and phosphates over the preference of secreted CKXs. This is likely to be a reflection of the prevalence of particular cytokinin derivatives within each intracellular compartment and illustrates that cytokinin concentrations are actively managed throughout the cell as well as throughout the plant.

When AtCKX genes were constitutively overexpressed in Arabidopsis or tobacco plants, all had an adverse impact on the phenotype, although diverse cytokinin deficiencies were seen (Werner et al., 2001, 2003). Overproduction of AtCKX1 and AtCKX3 caused the most severe phenotype with dwarfed aerial, vegetative tissues and very low fertility. Clearly the vacuolar pool of cytokinin nucleotides and glucosides is critical. Depletion by vacuolar CKXs appears to act as a net sink of cytokinins suggesting that the vacuolar pool could be actively managed. Strict compartmentation of cytokinin biosynthesis to plastids and cytokinin degradation to other major compartments is understandable, although the need for multiple sites for degradation complicates the picture. The full physiological significance of the high ability of vacuolar and cytoplasmic CKXs to cleave cytokinin metabolites remains so far unclear. Elucidation of the role of unique cytosolic CKX isoenzyme in cytokinin homeostasis is a challenging task considering its substrate specificity and almost constitutive and abundant pattern of expression (Smehilová et al., 2009; Vyroubalová et al., 2009).

Table 3

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Electron acceptor</th>
<th>pH</th>
<th>$k_{cat}$ (1/s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtCKX1</td>
<td>250 μM tZTP</td>
<td>500 μM ferricyanide</td>
<td>5.0</td>
<td>21.4</td>
<td>This work</td>
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<tr>
<td>AtCKX2</td>
<td>150 μM tZ</td>
<td>500 μM Qo</td>
<td>7.0</td>
<td>13.6</td>
<td>Calculated from Frébortová et al. (2007)</td>
</tr>
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<td>AtCKX3</td>
<td>250 μM iPR</td>
<td>500 μM ferricyanide</td>
<td>5.0</td>
<td>1.2</td>
<td>This work</td>
</tr>
<tr>
<td>AtCKX7</td>
<td>250 μM iP9G</td>
<td>500 μM ferricyanide</td>
<td>5.0</td>
<td>17.9</td>
<td>This work</td>
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<tr>
<td>ZmCKX1</td>
<td>150 μM iP</td>
<td>500 μM Qo</td>
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<td>143.6</td>
<td>Frébortová et al. (2004)</td>
</tr>
<tr>
<td>ZmCKX10</td>
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<td>100 μM ABTS radical</td>
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<td>258.7</td>
<td>Calculated from Frébortová et al. (2010)</td>
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<td>ZmCKX10</td>
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<td>250 μM ferricyanide</td>
<td>6.5</td>
<td>0.84</td>
<td>Calculated from Smehilová et al. (2009)</td>
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</table>

Fig. 4. Depletion of cytokinin phosphates from dehydrogenase reaction of (A) AtCKX1 (11 μg), (B) AtCKX3 (4 μg) and (C) AtCKX7 (12 μg). The decrease in concentration of initial 100 μM solution of each cytokinin in 50 mM MES/Tris buffer pH 5.0 in the presence of 500 μM Qo was followed by capillary electrophoresis. tZ and tZ phosphates were impossible to separate.
The $k_{cat}$ values for each of the AtCKX enzymes has been collated from this dataset and published data for plastic AtCKX2 and ZmCKX1 and cytosolic ZmCKX10 (Table 3). Vacuolar AtCKX3 showed very low $k_{cat}$ compared to other enzymes, even though all the values were determined under the best known conditions. However it is possible that an alternative electron acceptor or cytokinin is preferred. For example, a recent finding that ZmCKX1 has very high $k_{cat}$ in the presence of ABTS radical (Frébortová et al., 2010) made us wonder if an unknown, vacuole-specific electron acceptor might exist for these enzymes. It is known that radicals can be found in vacuoles, for example the oxidation of phenolic components in peroxidase-dependent reactions produces phenoxyl radicals, which are then reduced by ascorbic acid to form other radicals as intermediates (Takahama, 2004). It is clear that there is still more to learn about cytokinin homeostasis.

4. Experimental

4.1. Plant material

Transgenic tobacco plants overexpressing AtCKX1 (At2g41510) and AtCKX3 (At5g56970) genes from A. thaliana were prepared as reported previously (Werner et al., 2001). Tobacco AtCKX7 (At5g21482) overexpressor was prepared by the same procedure (Werner et al., unpublished). Plants were cultured in a greenhouse with 15-h light/9-h dark cycles, at 25 °C. Leaf material was harvested before flower induction, frozen immediately in liquid nitrogen and stored at −80 °C.

4.2. Isolation of AtCKX genes

The starting material for RNA isolation were leaves of transgenic tobacco plants overexpressing the genes of interest (Werner et al., 2001). RNA was isolated with Plant RNA 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 for each gene were designed so that resulting amplicons would be missing predicted N-terminal signal sequences [SignalP 3.0 Server, Bendtsen et al., 2004; Fig. 1]. Genes were amplified with the use of Phusion DNA Polymerase (Finnzymes, Espoo, Finland). A TGradient Thermocycler (Biometra, Göttingen, Germany) was programmed as follows: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 60 s at 60/65/60 °C (AtCKX1, AtCKX3 and AtCKX7, respectively), 30 s at 72 °C, and terminated by 10 min at 72 °C. Genes encoding vacuolar enzymes were further cloned into pGAPZα shuttle vector (Invitrogen), containing the S. cerevisiae α-factor secretion signal, and plasmid constructs were transformed into E. coli TOP10F (Invitrogen) by electroporation at 1.8 kV. Selection of transformants was based on zeocin resistance. AtCKX7 was cloned into pPICZA shuttle vector (Invitrogen). All primer sequences are shown in Supplementary Table 1.

4.3. Preparation of pGAPZα vector with an additional His-tag before multiple cloning site

To prepare pGAPZα vector containing an additional polyhistidine domain, the pET16b vector (Novagen, Madison, WI, USA) was used. The pET16b vector carries an N-terminal His-Tag sequence and this was precloned into pGAPZα. The resulting pGAPZαX(His)10 vector was used as an expression vector for vacuolar AtCKX proteins in P. pastoris X-33.

4.4. Production of recombinant AtCKX enzymes in P. pastoris

The plasmid constructs pGAPZα::AtCKX were linearized with AvrII (NEB, Ipswich, MA, USA) and used for integration into the P. pastoris X-33 (Invitrogen) genome. Yeast transformation was done by electroporation at 1.5 kV according to the manufacturer’s protocol. The transformants were selected on YPD plates (1% yeast extract, 2% peptone, 2% d-glucose, 1 M sorbitol, and 2% agar) containing 100 mg/l zeocin (Duchefa Biochemie B.V., Haarlem, The Netherlands). Selected transformants were picked up and grown for 1 day in 2 ml of YPD medium (2% peptone, 1% yeast extract, and 2% glucose) with 100 mg/l zeocin, at 30 °C with extensive shaking at 230 rpm. The pGAPZα::AtCKX transformants were then transferred into 50 ml of YPD medium without zeocin buffered to pH 7.2 with 0.1 M potassium phosphate buffer and cultivated for 48 h at 28 °C with 230 rpm shaking. After that yeast cells were removed by centrifugation at 5000g for 10 min and the CKX activity was measured in the cell-free supernatant.

The pPICZA::AtCKX7 plasmid construct was linearized with ScaI (Takara, Kyoto, Japan) and used for integration into the P. pastoris X-33 genome as described above. Sixty milliliters of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 × 10⁻⁵ M biotin and 1% glycerol) in 250 ml flask was inoculated by an overnight culture of P. pastoris pPICZA::AtCKX7 (grown with 100 mg/l of zeocin) and cultivated for 16–18 h at 30 °C and 180 rpm. The cells were harvested by centrifugation, resuspended to OD₆₀₀ of 1 in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 × 10⁻⁵ M biotin and 0.5% methanol), divided to 200 ml aliquots and cultivated in 500 ml flasks at 28 °C and 230 rpm. After 22 h the culture medium was supplemented with MeOH to a final concentration of 0.5%, the cells were cultivated for next 4 h and then harvested by centrifugation. Pichia cells were resuspended in breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol) and disrupted by vortexing with an equal volume of acid-washed glass beads (425–600 μm, Sigma). After centrifugation activity was measured in the cell lysate.

4.5. Production of recombinant AtCKX7

The ORF of AtCKX7 was subcloned into the pTBY12 vector through Ndel and EcoRI restriction sites (NEB) and transformed into E. coli BL21 (DE3) STAR (Invitrogen). The transformant culture was diluted to OD₆₀₀ = 0.1, grown at 22 °C until it reached OD₆₀₀ = 0.5 and subsequently induced for 5 h at 18 °C with 0.5 mM IPTG. The cells were collected and resuspended in 60 ml of 0.02 M Tris/HCl (pH 8.0), 0.1% Triton-X 100, 500 mM NaCl and 1 mM EDTA and disrupted by a French press (20,000 psi) (Thermo, Waltham, MA, USA). The lysate was centrifuged and the supernatant purified via chitin affinity chromatography. Twenty ml of chitin resin (NEB; 5 × 1.6 cm) was equilibrated in 0.02 M Tris/HCl (pH 8.0), 0.1% Triton-X 100, 500 mM NaCl and 1 mM EDTA. The sample was loaded onto the column and washed with 80 ml of equilibration buffer. Subsequently, 60 ml of equilibration buffer supplemented with 50 mM DTT was passed through the column to induce intein tag cleavage and the sample was incubated at 16 °C for 62 h. AtCKX7 protein was then eluted with 50 ml of equilibration buffer without DTT. The elution fraction was concentrated on Amicon centrifugal cellulose filter with cut-off 10 kDa (Millipore) and stored at −20 °C.

4.6. Preparation of pPIC9K vector under control of constitutive GAP promoter

pPIC9K vector (Invitrogen) and proper plasmid constructs pGAPZαX(His)10::AtCKX were subjected to partial digestion with...
and the proteins were then eluted by a linear gradient of 10 mM potassium phosphate buffer (pH 7.6)/C2 and eluate was fractionated. The fractions showing enzyme activity were finally purified on a Ni Sepharose HP (GE Healthcare; 18 cm) connected to BioLogic Duo-Flow FPLC system equipped with UV and conductivity detector (Bio-Rad). Fractions with CKX activity were pooled, concentrated by ultrafiltration and stored at ~20 °C.

Protein content in enzyme samples was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

4.8. SDS–PAGE and immunoblot analysis

Tricine–SDS–PAGE was performed on a slab gel (8%) in Tris–tricine running buffer according to Schägger and von Jagow (1987). Recombinant molecular weight standard mixture (Sigma) was used as a marker. Protein samples were heated before application at 100 °C for 10 min in the presence of 1% SDS and 1% 2-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany) or proteins were blotted onto PVDF membrane (0.45 μm) in the MiniTrans blot system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% powdered milk in 20 mM Tris/HCl (TBS buffer, pH 7.6) for at least 1 h, washed with TBS containing 0.1% of Tween-20 (TWEEN-TBS buffer) and incubated for at least 1 h in TBS containing 1% powdered milk and a rabbit polyclonal antibody raised against the C-terminal fragment of barley HvCKX1 or HvCKX2 (Galuszka et al., unpublished results). The membrane was subsequently washed four times with Tween-TBS and incubated for 1 h with anti-rabbit IgG horseradish peroxidase conjugate (Sigma) in TBS containing 1% powdered milk. After rinsing in Tween-TBS buffer, membranes were developed with Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK) and Lumi-film chemiluminescent detection film (Roche, Basel, Switzerland) according to the manual.

4.9. Identification of recombinant proteins by MALDI-TOF mass spectrometry

Protein bands (containing picomolar protein amounts) were excised from Coomassie-stained SDS–PAGE gels. MALDI-TOF peptide mass fingerprinting was conducted after a previous in-gel digestion of samples by modified trypsin (Šebela et al., 2006). The digestion protocol additionally involved reduction and alkylation steps (Shevchenko et al., 2007) and proceeded overnight at 37 °C. The instrument used was a Microflex LRF20 MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a microScout ion source and a 337-nm nitrogen laser (10 Hz). Measurements...
were performed in the reflectron mode for positive ions. Parameters of the instrument were as follows: an acceleration voltage of 19 kV, an extraction voltage of 16.1 kV, a lens voltage of 9.1 kV, a reflectron voltage of 20 kV and a delayed extraction of 250 ns. Digest aliquots (0.6 µl) were pipetted onto an MSP AnchorChip™ 600/96 target plate, overlaid with 0.6 µl of a matrix solution (5 mg α-cyano-4-hydroxycinnamic acid in 0.33 ml of 2.5% v/v trifluoroacetic acid and 0.66 ml acetonitrile) and left to dry in air. Mass spectra were accumulated from 100 to 200 laser shots, the examined m/z range was 500–6000; a mixture of peptide standards (Bruker Daltonik) was used for external calibration. The acquired spectra were processed by flexAnalysis 2.4 and Biotools 3.2 software (Bruker Daltonik). Database searches were performed against Swiss-Prot (release 57.12) and NCBI nr (release December 15, 2009) databases using the program Mascot Server 2.2 (Matrix Science, London, UK). As variables, oxidation of methionine and carbamidomethylation of cysteine plus one missed cleavage were chosen for all searches performed without taxonomic restriction; a mass tolerance of 150 ppm was allowed. Post-source decay (PSD) spectra of selected peptides were recorded in 15–17 segments, with each succeeding segment representing a proportional reduction in reflectron voltage. About 300–500 laser shots were averaged per segment. All segments were pasted, calibrated, and smoothed to a final spectrum under computer control by the flexAnalysis 2.4 software.

4.10. CKX activity assay

For determination of electron acceptor preference of AtCKX proteins, the enzymatic activity was assayed with 250 µM IPR as a substrate without the acceptor (in the presence of oxygen) and with the use of following electron acceptors (500 µM): 2,6-dichlorophenol indophenol (DCPIP; LOBA Feinchemie, Fischamend, Austria), 2,3-dimetoxy-5-methyl-1,4-benzoquinone (Q0; Sigma, St. Louis, MO, USA), ferricyanide (Lachema, Brno, Czech Republic), 3’,5’-dimethoxy-4-hydroxyacetophenone, cytochrome c, 2,3,5-triphenyl-tetrazolium chloride (all from Sigma), NAD+ (Fluka, Buchs, Switzerland). For the assessment of substrate specificity 500 mM each and the enzyme sample. The reaction mixture aliquots without stopping the reaction. All the experiments were performed using an Agilent 3D CE System (Agilent Technologies, Waldbronn, Germany) equipped with a diode array UV–Vis detector. Experimental data were collected and analyzed with 3D CE ChemStation Software. Separations were carried out in bare-fused silica capillaries (PolymerMicro Technologies, Phoenix, AZ, USA). Total length of the capillary was 64.5 cm (56 cm effective), 50 µm I.D. A new capillary was flushed with 1 M NaOH (15 min), deionized water (15 min) and equilibrated with background electrolyte (20 min). After each run the capillary was rinsed with 1 M NaOH (1 min), deionized water (1 min) and background electrolyte (2 min). Basic running buffer was used and the analytes were separated as anions in positive mode. Separations were carried out at 30 kV, the capillary was thermostatted to 20 °C. All quantifications were done at 268 nm using corrected peak areas and uridine monophosphate as an internal standard.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.08.013.

References


