

# Cytokinin affinity purification and identification of a tobacco BY-2 adenosine kinase

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**Abstract** Adenosine kinase is one of the enzymes potentially responsible for the formation of cytokinin nucleotides in plants. Using a zeatin affinity column a 40 kDa protein was isolated from tobacco Bright Yellow 2 (TBY-2) and identified by mass spectrometry as adenosine kinase. The ligand interaction reported here can be disrupted by several other adenine- but not guanine-based purine derivatives. The observed interaction with cytokinins is discussed in view of a putative role for adenosine kinase in TBY-2 cytokinin metabolism. The presented results show for the first time a plant adenosine kinase affinity-purified to homogeneity that was identified by primary structure analysis.

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**Key words:** Adenosine kinase; Tobacco Bright Yellow 2; Cytokinin; Protein identification

## 1. Introduction

The involvement of cytokinins is one of the particular features in the regulation of the plant cell cycle. In early years documented as a group of hormonal regulators promoting cell division, cytokinins appeared later to play major roles in various stages of plant growth and development. The cytokinin autonomous and highly synchronisable plant cell suspension culture tobacco Bright Yellow 2 (TBY-2) [1] is an excellent tool for dissecting cell cycle events and has rapidly enhanced insights into the way cytokinins control the cell cycle. In this model organism zeatin-type (Z-type) cytokinins accumulate at G2-M and G1-S transition [2] and modulate further cell cycle progression, at both transitions in a distinct way [3,4]. Briefly, after the elevated zeatin (Z, Fig. 1) and zeatin riboside (ZR) content, transient accumulations of zeatin riboside-5'-monophosphate (ZRP) were observed. Whereas Z and ZR are generally considered as the active forms, it remains unclear whether the nucleotide form is a precursor or a conversion product of the existing cytokinin pool. In the latter case it may represent an intermediary form of an interconversion

reaction, an inactivated form, or an active form with a thus far unidentified function.

A few enzymes are potentially responsible for the formation of ZRP [5,6]. A first candidate is isopentenyltransferase (IPT), which catalyses the formation of isopentenyladenosine-5'-monophosphate from adenosine-5'-monophosphate and isopentenylpyrophosphate. Furthermore, a recently characterised isoform of IPT transfers the isopentenyl group to ATP and ADP [7]. The IPT enzyme is responsible for the de novo biosynthesis of cytokinin nucleotides that can be converted to various cytokinin nucleosides, bases and other metabolites. Two other enzymes have the ability to convert the existing cytokinin pool to cytokinin nucleotides. Both are enzymes generally involved in adenine salvage processes. Adenine phosphoribosyltransferase can form cytokinin nucleotides from the corresponding base [8–10]. Adenosine kinase (ADK) can phosphorylate cytokinin nucleosides to their corresponding nucleotides [11,12].

Phosphorylation of cytokinins can have serious physiological implications [13]. Exogenously administered isopentenyladenosine (iPA) induced apoptosis in TBY-2 cells via a caspase-dependent pathway. Blocking ADK with 4-amino-3-iodo-1(β-D-ribofuranosyl)pyrazolo[3,4-d]-pyrimidine, a specific inhibitor, drastically reduced apoptosis, pointing towards ADK-dependent phosphorylation as a prerequisite in this phenomenon.

Considering both the accumulation of ZRP in the TBY-2 cell cycle and the cytotoxic effect of isopentenyladenine (iP)-type cytokinin nucleotides on TBY-2, the cytokinin phosphorylation becomes a physiologically relevant pathway. This paper focusses further on this phenomenon with the identification of ADK. Understanding to what extent this enzyme is involved in the formation of cytokinin nucleotides may reveal new insights into plant cell growth regulation.

ADK was previously purified and cloned from a human source [14]. In plants two isoforms from *Arabidopsis* [15] and one from the moss *Physcomitrella* [16] were cloned. Three reports describe the purification of ADK from a plant source [12,17,18], but the isolated plant protein was never structurally analysed. We report here the cytokinin-based affinity purification of TBY-2 ADK and its identification through primary structure analysis.

## 2. Materials and methods

### 2.1. Chemicals, cell culture and sampling

All chemicals were purchased from Sigma (Bornem, Belgium) un-

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**Abbreviations:** ADK, adenosine kinase; DMF, dimethylformamide; iP, isopentenyladenine; iPA, isopentenyladenosine; IPT, isopentenyltransferase; TBY-2, tobacco Bright Yellow 2; Z, zeatin; ZR, zeatin riboside; ZRP, zeatin riboside-5'-monophosphate

less stated otherwise. The TBY-2 culture was maintained as described [1] in Murashige and Skoog-based medium (Duchefa, Haarlem, The Netherlands) on a rotary shaker (130 rpm) at 27°C in darkness, and weekly diluted 50-fold in 50 ml of fresh medium. Samples for protein extraction were taken from an exponential culture. This was obtained by transfer of 17.5 ml of 7-day-old (stationary) culture to 250 ml of fresh medium in a 1-l Erlenmeyer and maintained under standard culture conditions for 2 days. Samples of 50 ml were taken and filtered over a Whatman no. 1 filter paper on a vacuum bottle. The cell pellet was scraped off and transferred to liquid nitrogen. Samples were either used immediately or stored at –70°C until the extraction.

## 2.2. Protein extraction

Soluble protein fractions were obtained by grinding the sample with a mortar and pestle in liquid nitrogen and 10 ml glycerophosphate buffer (50 mM  $\beta$ -glycerophosphate, 20 mM ethylene glycol-bis[ $\beta$ -aminoethyl ether]-*N,N,N',N'*-tetraacetic acid, 5 mM NaF, pH adjusted to 7.4 with NaOH), supplemented with 15 mM MgCl<sub>2</sub>, 500  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ M NH<sub>4</sub>MoO<sub>4</sub>, 1% (w/v) polyvinylpyrrolidone, 2 mM dithiothreitol and 1% (v/v) plant protease inhibitor cocktail. After thawing, the extracts were cleared by centrifugation (30 min at 6500 $\times$ g) and filtered at 0.45  $\mu$ m. Protein yield was typically 1.5 mg/ml, determined as described by Bradford [19] with bovine serum albumin to make a standard curve.

## 2.3. Preparation and testing of the affinity matrices

Epoxy-activated Sepharose 6B (containing 20–40  $\mu$ mol of epoxy-activated groups in 1 ml of drained gel) was swollen for 2 h in water that was refreshed three times. Prior to the coupling reaction, the resin was washed with 0.1 M Na<sub>2</sub>CO<sub>3</sub>. The coupling reaction was performed using 60  $\mu$ mol *trans*-Z, dissolved in a mixture of 4 ml dimethylformamide (DMF) and 4 ml 0.1 M Na<sub>2</sub>CO<sub>3</sub>. The activated resin was divided into 2-ml aliquots and mixed with the Z solution, followed by 24 h shaking on ice. After incubation, the resin was washed with water and mixed with 10 ml of 1 M ethanolamine, pH 9.0. The blocking reaction was shaken for 6 h at room temperature. The prepared affinity resin was sequentially washed with water, 50% (v/v) DMF in water, 0.1 M Na<sub>2</sub>CO<sub>3</sub> and glycerophosphate buffer. The prepared affinity resin was checked by measuring the UV spectrum and stored at 4°C in glycerophosphate buffer containing 0.2% NaN<sub>3</sub>. The functionality of the prepared affinity resin was verified with polyclonal antibodies directed against Z [20]. In brief, an aliquot of the resin was incubated with the antibodies and the resin was rinsed with glycerophosphate buffer. The antibodies were then eluted with 100 mM glycine hydrochloride, pH 2.5 and collected in concentrated phosphate-buffered saline. By means of a radioimmunoassay the cytokinin binding activity was detected in the eluate, confirming the suitability of the prepared resin for protein isolation.

## 2.4. Cytokinin affinity purification

Soluble extracts were subjected to cytokinin affinity chromatography on 100  $\mu$ l (bed volume) Z-Sepharose columns. To each column 2.5 ml of cell extract was applied. The columns were washed with 100 bed volumes glycerophosphate buffer. The proteins of interest were eluted by overnight incubation of the affinity matrix using 1 ml glycerophosphate buffer supplemented with 2 mM competitor (see Section 3). After elution the columns were washed sequentially with 1 ml 10 mM glycine-HCl (pH 1.7), 4 ml 6 M urea and 3 ml glycerophosphate buffer. The columns were stored in glycerophosphate buffer containing 0.2% NaN<sub>3</sub>.

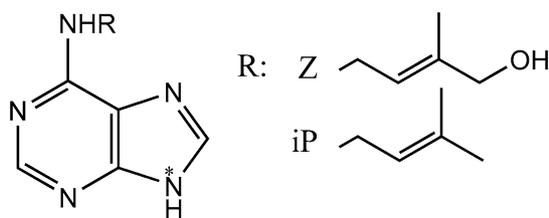


Fig. 1. Structure of the cytokinins zeatin (Z) and isopentenyladenine (iP). Z was immobilised via a spacer attached to the marked (\*) position. This is also the position where the riboside or ribotide is bound in the case of cytokinin nucleosides or nucleotides.

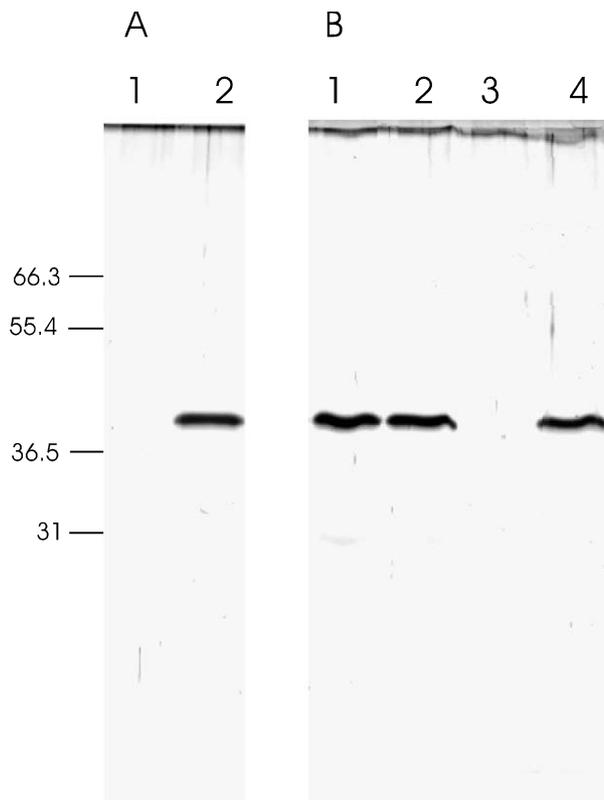


Fig. 2. SDS-PAGE of Z affinity-purified TBY-2 proteins. Soluble proteins from TBY-2 were subjected to Z affinity chromatography. A: Proteins were eluted with buffer (A1) or buffer with 2 mM Z (A2). B: The effect of elution with 2 mM of various nucleosides was tested with iPA (B1), adenosine (B2), guanosine (B3), and ZR (B4). Positions of molecular weight marker proteins (kDa) are indicated on the left.

## 2.5. Electrophoresis

Prior to electrophoresis the proteins were precipitated by addition of trichloroacetic acid up to 10% (w/v). After 30 min of incubation at 4°C the samples were centrifuged (10 min at 15000 $\times$ g), the supernatant was discarded and the protein pellet was washed with ethanol: ether (1:1, v/v). After drying the protein pellet was incubated at 95°C for 5 min in 50  $\mu$ l 62.5 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol. Fractions were analysed by SDS-PAGE on lab-cast gels (12% T). Mark 12 (Invitrogen, Merelbeke, Belgium) was used as a molecular weight standard. Separated proteins were silver-stained according to [21].

## 2.6. Tryptic digestion and ESI-QTOF mass spectrometry

Protein bands of interest were cut from the gels and subjected to in-gel tryptic digestion as described [21]. The resulting peptide mixture was analysed by nano-electrospray quadrupole time of flight mass spectrometry (QTOFII, Micromass, Manchester, UK) according to Wilm et al. [22]. The obtained spectra were interpreted using the accompanied Masslynx software and identified by submission to various public databases using the Mascot search engine [23]. Unannotated expressed sequence tag (EST) hits were further submitted for identification by performing Blast searches using the obtained peptide fragment sequences only [24].

## 3. Results

### 3.1. Zeatin affinity chromatography with soluble TBY-2 protein fractions

In order to isolate proteins interacting with cytokinins *trans*-Z (Fig. 1) was immobilised on a Sepharose 6B resin. Soluble protein extracts obtained from TBY-2 were applied

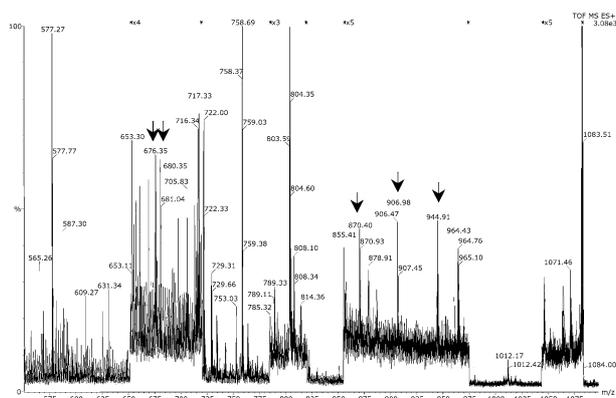


Fig. 3. Survey scan spectrum of the isolated protein. Diagnostic peptide masses are shown in magnified portions of the spectrum.

directly to this matrix and the resin was washed until no proteins were detected in the eluate. When the resin was incubated with 2 mM Z, the eluate contained a clear protein band with an estimated molecular weight of 40 kDa under denaturing conditions (Fig. 2). A control treated in the same way without Z in the elution step did not yield detectable proteins. Isoelectric focusing (pH 3–10) of the eluted protein fraction prior to SDS-PAGE did not further separate this protein into distinct spots (data not shown), indicating its isolation to homogeneity.

### 3.2. Identification as ADK

After tryptic cleavage the digest was subjected to nano-ESI mass spectrometry analysis. Submission of the peptide mass fingerprint list (Table 1) obtained by the survey scan (Fig. 3) to the SwissProt and NCBI nr databases using the Mascot search engine did not result in significant identification hits. Submission of fragmentation spectra of five selected peptides did not result in a significant outcome either. Only querying the peptide sequences against the EST database yielded significant identity scores. Since the obtained DNA clones were not annotated with a protein homologue the peptide sequences were used for a protein BLAST. This resulted in the identification of the isolated protein as ADK. Each of the retrieved cDNAs covered some of the five peptide tags. One of the five sequenced fragments (*m/z* 870.40) could only be assigned by introducing a K→R mutation mass shift (Fig. 4). In silico digestion of the four cDNA sequences representing four different families used for identification yielded no single common tryptic peptide tag (mass > 640 Da). By means of sequence homology assembly across the different species we could cover a larger part of the conserved ADK sequence (Fig. 4).

Table 1

Tryptic peptide fingerprint *m/z* and experimental monoisotopic mass (in parentheses) peptide list for the purified protein

<i>z</i>	<i>m/z</i> (experimental monoisotopic mass)	
2	<b>676.32</b>	705.83
	(1350.64)	(1409.66)
3	<b>680.35</b>	772.70
	(2038.05)	(2315.1)
4	789.09	814.33
	(3152.36)	(3253.32)

Corresponding sequences from sequenced peptides (bold) are also shown. 676.32: AGCYASNVIQR (C = carboxymethylcysteine), 680.35: ITVITQGADPVVVAEDGKVK, 870.40: FNVEYIAGGATQNSIR, 906.46: ITVITQGADPVVVAEDGK, 944.41: VLPYMDVVFNGNETEAR.



Fig. 4. ClustalW alignment of protein sequences matching the isolated protein. The gi numbers correspond to *Arabidopsis thaliana* ADK2 (gi117366963), *Arabidopsis thaliana* ADK1 (gi117367081), *Physcomitrella patens* ADK (gi2661840), a *Lycopersicon esculentum* EST (gi15196656) and a *Gossypium arboreum* EST (gi13245888) respectively. Sequenced peptides from the TBV-2 protein are also shown (shaded black). Only the relevant part of the EST sequences is included. Identical residues: black; similar residues: dark grey; other residues: light grey.

### 3.3. Effect of structural analogues on the interaction of ADK with immobilised zeatin

The effect of various nucleosides on the interaction of the identified ADK with immobilised Z was tested. Therefore the purification was repeated using 2 mM each of ZR, adenosine, guanosine and iPA in the elution step (Fig. 2B). All of the adenine-based nucleosides eluted ADK under the given conditions with a more or less similar efficiency. In contrast, guanosine failed to elute ADK from the column.

## 4. Discussion

In this paper we describe the purification of ADK from the TBV-2 culture. Reports citing the successful purification of ADK from plant sources are scarce, and based on the combination of sequential chromatographic steps [12,17,18]. In contrast to these studies we prepared an affinity matrix to which a homologue of its putative substrate was immobilised. This affinity-based purification strategy drastically speeded up the isolation procedure. Currently available mass spectrometric techniques enabled us to identify this protein on the level of its primary structure. To our knowledge this is the first time an ADK protein from a plant source was identified by means of primary structure analysis. In previously published purifi-

cations of plant ADK, its identity was only demonstrated by activity measurements.

Although the cytokinin-based affinity purification procedure here employed could have led to the purification of downstream cytokinin targets, virtually no other proteins were detected in the eluate. A tentative explanation is that ADK impedes the binding of other proteins by occupying the available cytokinin ligands on the column. An alternative possibility is that the isolation of cytokinin target proteins requires a different extraction procedure or affinity matrix than used in these experiments.

The purification strategy used here shows that TBY-2 ADK interacts directly with the free base Z. This means that the presence of a ribose group is not absolutely required for binding, although the base form is not subject to phosphate transfer. At the concentrations used the tested adenine nucleosides could easily elute ADK from the Z resin, this in contrast to guanosine, which had almost no effect. This is in accordance with previous data showing that ADK appears to be specific for an adenine-based purine ring [25]. Most likely the Z interaction occurs via the nucleoside substrate binding site of ADK.

The observation of a direct interaction of ADK with cytokinins is very interesting in relation to cytokinin interconversion reactions. Both iP and Z-type cytokinins were able to elute the ADK, pointing to this enzyme as a good candidate for being responsible for cytokinin phosphorylation in TBY-2. The relevance of iPA phosphorylation by ADK has already been shown [13]. As shown earlier by our group the formation of ZRP appears to follow the increase in the content of non-phosphorylated cytokinins during the TBY-2 cell cycle. This succession supports the hypothesis that an adenine salvage enzyme such as ADK, rather than the de novo cytokinin nucleotide synthesising enzyme IPT, is involved.

In *Arabidopsis thaliana* two ADK isoforms are known [15]. To answer the question whether more than one isoform is present in the purified fraction from TBY-2, two-dimensional electrophoresis with this eluate was performed (data not shown). Only one spot could be detected. If, as in *Arabidopsis*, more than one isoform of ADK is expressed in growing TBY-2 cells, the fact that only one protein is isolated suggests that there is a difference in the specificity of the isoforms for adenine derivatives. Analysis of the peptide sequences from TBY-2 ADK did not show significantly higher similarity to either one of the *Arabidopsis* ADK isoforms. The next stage will be cloning the gene for TBY-2 ADK and its potential isoforms. Functional analysis will subsequently make it possible to further describe the role of ADK in the cytokinin metabolism of TBY-2.

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