Identification of Four Adenosine Kinase Isoforms in Tobacco By-2 Cells and Their Putative Role in the Cell Cycle-regulated Cytokinin Metabolism*

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Adenosine kinase (ADK), a key enzyme in the regulation of the intracellular level of adenosine is also speculated to be responsible for the conversion of cytokinin ribosides to their respective nucleotides. To elucidate the role of ADK in the cytokinin metabolism of tobacco BY-2 cells (Nicotiana tabacum cv. “Bright Yellow-2”; TBY-2), we have identified and characterized the full-length cDNAs encoding four ADK isoforms of N. tabacum and determined their catalytic properties. The four TBY-2 ADK isoforms (designated 1S, 2S, 1T, and 2T) display a high affinity for both adenosine (K_m 1.88–7.30 μM) and three distinct types of cytokinin ribosides: isopen- tenyladenosine; zeatin riboside; and dihydrozeatin riboside (K_m 0.30–8.71 μM). The V_max/K_m values suggest that ADK2S exhibits in vitro an overall higher efficiency in the metabolism of cytokinin ribosides than the other three isoforms. The expression pattern of NTADK genes is modulated significantly during the cell cycle. We suggest that the increased transcript accumulation of NTADK coupled to an increased ADK activity just prior to mitosis is associated with a very active cytokinin metabolism at that phase of the cell cycle of synchronized TBY-2 cells.

Cytokinins play an essential role in almost all aspects of plant growth and development including cell division, shoot initiation and growth, leaf senescence, and photomorphogenesis (1, 2). Active cytokinins contain an adenine moiety substituted at N^6 with an isoprene derivative or an aromatic ring. Their pool is controlled in plants by the rate of import, biosynthesis, inactivation, and degradation (3). One of the major steps in cytokinin metabolism is the conversion of free bases and ribosides to nucleotides. The latter is proposed to be one of the inactive forms of cytokinins (4–6). The cytokinin ribosides and their nucleotides are the most abundant naturally occurring cytokinins (4, 7–10). It has been reported that external application of cytokinin bases and ribosides is associated with rapid formation of the corresponding nucleotides (11). Therefore, it seems probable that phosphorylation is one of the metabolic pathways that plants use to preserve the optimally balanced levels of active cytokinins, essential for normal growth and development. This mechanism by which plants modulate the amount of various forms of cytokinins is proposed to involve adenosine kinase (ADK), a key enzyme in the regulation of the intracellular level of adenosine (ADO) (12–14).

ADK has been characterized in many eukaryotic species (12–18) and recently also in the prokaryote, Mycobacterium tuberculosis (19). In an attempt to identify proteins that specifically interact with zeatin (Z), Laukens et al. (20) isolated a 40-kDa protein from tobacco BY-2 (TBY-2, Nicotiana tabacum cv. “Bright Yellow-2”) cell extract further identified by mass spectrometry as a putative ADK.

The cytokinin autonomous and highly synchronizable TBY-2 cell suspension culture (21) is an excellent tool for the investigation of the cell cycle events. Examinations of endogenous cytokinins fluctuations during the cell cycle published by our group (22) revealed transient accumulation of particularly zeatin-type cytokinins at the end of S phase, during G2-M transition, and during G1 phase. A transient peak of Z (23) has been reported to be essential for G2-M transition as the concurrent blocking of cytokinin accumulation and mitosis by lovastatin, an inhibitor of cytokinin biosynthesis (24), could be specifically reversed by the application of exogenous Z (23).

In this paper, we report the cloning and characterization of full-length cDNAs encoding four ADK isoforms of N. tabacum. The catalytic properties of tobacco ADKs and the kinetics of the ADK activity during the cell cycle progression of TBY-2 cells point to an active participation of ADK in the cytokinin metabolism. To our knowledge, this is the first report that links ADK with tobacco BY-2 cell division.

EXPERIMENTAL PROCEDURES

Plant Materials—Nicotiana sylvestris, Nicotiana tomentosiformis (seeds kindly provided by R. M. Maier, Ludwig-Maximilians-Universität, München, Germany), and N. tabacum seeds were sown in Petri dishes on solidified Murashige and Skoog medium supplemented with

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AJ895805, AJ895806, Y895651, Y895655, AJ895644, AJ895652, and AJ895653.

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5 The abbreviations used are: ADK, adenosine kinase; ADO, adenosine; IP, isopentenyladenosine; iPA, isopentenyladenine; iPMP, isopentenyladenosine monophosphate; Z, zeatin; ZR, zeatin riboside; ZMP, zeatin riboside monophosphate; DZ, dihydrozeatin; TBY-2, tobacco “Bright Yellow-2”; AFLP, amplified fragment-length polymorphism; RNAi, RNA interference.
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20 g/liter sucrose. The seedlings were cultivated in a growth chamber at 24 °C with 8-h dark/16-h light cycles.

Cell Culture Maintenance and Synchronization—BY-2 cells were maintained and synchronized as described by Nagata et al. (21). The stationary culture was transferred in a proportion of 1:10 to the fresh medium (Murashige and Skoog medium, 30 g/liter sucrose, 200 mg/liter KH₂PO₄, 1 mg/liter thiamine, and 0.2 mg/liter 2,4-dichlorophenoxyacetic acid) containing 5 mg/liter aphidicolin (ICN Biomedicals, Asse, Belgium). The culture was agitated on a rotatory shaker at 27 °C and 130 rpm in the dark. After 24 h of incubation, the cells were extensively maintained with medium and then resuspended in fresh medium.

Cells were fixed in ethanol/acetic acid solution (3:1, v/v) and stained with 4',6-diamidino-2-phenylindole (1 μg/ml). The mitotic index was determined by scoring the nuclei in mitosis under fluorescence microscope (Nikon).

Library Screening—To increase the probability of isolation of all cDNAs for ADK, two available tobacco BY-2 cDNA libraries were screened, one representing genes expressed in exponential phase and a second one with genes expressed after methyl jasmonate treatment.² Both libraries were screened by hybridization to a 32P-labeled 335-bp probe (Rediprime TM II, Amersham Biosciences, and Bio-Spin chromatography columns, Bio-Rad, Nazareth Eke, Belgium) obtained by reverse transcription-PCR with Titan One Tube reverse transcription-PCR SuperScript II Supplied Science, Belgium), using total RNA from tobacco leaf and primers Adk335F/R (Table I), the sequence of which was derived from a tomato EST database (TMECD65TV). Two cDNA libraries were used for the hybridization screening as described by Nagata et al. (21). The first library was isolated from tobacco BY-2 cell cultures from transgenic lines expressing the gymnostomum 1-thio-diaminopseudouridine 5′-pentenyl[2-3H]adenosine (an inhibitor of Ado deaminase) was included (Sigma). The reaction was carried out at 30 °C for 10 min and stopped by adding 100 μl of 0.25 M EDTA. The radiolabeled nucleotides formed during the reaction were separated from nucleosides on Supelclean LC-NH₂ SPE Tubes (Supelco, Bellfonte, PA) according to the manufacturer’s instructions. Samples were loaded on columns equilibrated with 20 mM Tris-HCl (pH 7.2). Subsequently, columns were washed with 12 washes of 20 mM Tris-HCl (pH 7.2). Negatively charged nucleotides were eluted with 2 volumes of 2.5 M NaCl and quantified by liquid scintillation counting (Tri-Carb 1500 scintillation analyzer, Packard BioScience, Zellik, Belgium). The values of Kᵣ and Vₘₐₓ were calculated by a non-linear regression with the aid of the program Hyper 1.1 (copyright J. S. Easterby).

PCR Cloning and Expression Analysis—Oligonucleotide primers for each of the cloned cDNAs were used to produce a total of 200 different oligonucleotides. One milliliter of each recombinant bacteria culture was centrifuged (10 min at 14,000 × g), and the cell pellet was resuspended in 100 μl of BugBuster containing 2.5 units of Benzonase (His Bind resin and buffer kit). SDS-PAGE was performed in a 12% (w/v) separation gel and 5% (w/v) stacking gel. Separated proteins were silver-stained according to Sambrook and Russell (34). In parallel, proteins were transferred to a polyvinylidene difluoride membrane using a Transblot apparatus (Novex, Invitrogen). The protein blot was probed according to the manufacturer’s protocol with monoclonal antibodies against His (Sigma) along with a secondary antibody, goat anti-mouse IgG AP conjugate (Novagen).

RNA Extraction, cDNA Synthesis, and Expression Analysis—Total RNA from plant material was isolated with the RNeasy kit (Ambion, Austin, United States) according to the manufacturer’s instructions. The RNA by total RNA from BY-2 cell culture samples collected at 11 time points during the cell cycle using TRIzol reagent (Invitrogen) and treated with DNase I (Ambion). DNA was synthesized from 2 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Ambion) with oligo(dT)₁₈ as primer.

Primers used in transcript abundance analysis amplified fragments of 942 bp of NaADK1 (Tor1F/Tor1R), 527 bp of NaADK2 (Tor2F/Tor2R), and 602 bp of ACTIN (ActinN2F/ActinN2R). The PCR products were analyzed by electrophoresis on a 1.5% Tri borate-EDTA-agarose gel and stained with SYBR Green. The relative abundance of generated fragments was quantified using a fluorescence imaging device (Typhoon 9400, Amersham Biosciences) and analyzed with ImageQuant imaging software (Molecular Dynamics).

Cytokinin Measurements—Frozen cell samples were ground in liquid nitrogen and extracted overnight at −20 °C in Bieskes buffer (32). Deuterated standards were added to the extracts as internal tracers. Cytokinins were purified on a combination of DEAE-Sephadex and RP-C₁₈ cartridges. The purified extracts were loaded onto immunofinity column containing monoclonal antibodies against cytokinins. After immunopurification, the cytokinins were analyzed by high pressure liquid chromatography linked to Quattro II mass spectrometer equipped with an electrospray interface (33). The chromatograms obtained were processed by means of Masslynx software (Waters, Zellik, Belgium), and the cytokinin concentrations were determined according to the principle of isotope dilution.

RESULTS

The Tobacco BY-2 Cell Line Harbors Four ADK Isoforms That Were Inherited from Its Ancestors in a Pairwise Manner—Two BY-2 cDNA libraries were screened with a 335-bp probe for putative ADK generated with primers whose sequence was derived from a tomato (Lycopersicum esculentum) EST data base (see “Experimental Procedures”). 23 clones were isolated and upon sequencing were found to correspond to four distinct cDNAs. BlastX comparison (27) of the determined nucleotide sequences against the NCBI non-redundant protein data base

² R. Vanderhaegen and A. Goossens, unpublished data.

³ Available at homepage.ntlworld.com/john.easterby/software.html.
revealed a striking homology to ADK genes. Thus, the isolated cDNA clones were designated as NtADKs. Furthermore, cDNA clones exhibited a two-by-two high sequence similarity. Nucleotide comparisons for each pair revealed identities of 95–96%.

N. tabacum is a natural allotetraploid derived from ancestors of Nicotiana sylvestris (the maternal S genome donor) and Nicotiana tomentosiformis (the paternal T genome donor) (35, 36). Hence, we presumed that the two pairs of N. tabacum homologues represent orthologous genes. Those two pairs were initially designated NtADK1 and NtADK2. To verify this presumption, we designed primer pairs Tor1F/R and Tor2F/R (Table I), annealing solely to identical regions within either pair of homologues. Amplification of reverse-transcribed mRNA isolated, respectively, from N. tomentosiformis leaf or N. sylvestris leaf with both primer pairs yielded four single amplification products of lengths consistent with those anticipated from the leaf with both primer pairs yielding four single amplification products of lengths consistent with those anticipated from the nucleotide sequences of the NtADK clones (942 bp of both NtADK1 clones amplified with Tor1F/R; 527 bp of both NtADK2 clones amplified with Tor2F/R). Subsequent sequencing and nucleotide sequence alignment revealed 99–100% identity between the individual PCR fragments and the NtADK clones and resulted in their assignment to NtADK-syl or to NtADK-tom (Fig. 1A, only partial alignment is presented).

The largest open reading frame of each clone begins with an AUG codon and ends with a TAA codon (NtADK1) or a TGA codon (NtADK2). Sequence analysis of tom-type NtADK clones revealed short (Sh) and long (Lg) transcript variants (1310 and 1422 bp of NtADK1-tom and 1258 and 1305 bp of NtADK2-tom) (Fig. 1B). The lengths of NtADK1-syl and NtADK2-syl were 1377 and 1307 bp, respectively.

The open reading frame of each clone contained 340 codons. For the purpose of discussion, the putative TBY-2 ADK isoforms have been designated as 1T, 1S, 2T, and 2S, respectively.4 The alignment over the full length of the predicted polypeptides is presented in Fig. 1C. The deduced amino acid sequences display an identity of 97% between the orthologous S- and T-type ADKs and an identity with ADKs reported of other species varying between 19–25% (M. tuberculosis) and 83–86% (A. thaliana). The assignment of four ADK isoforms to two groups designated ADK1 and ADK2 should not be related to classification of the two previously identified ADK isoforms of A. thaliana. The phylogenetic analysis of the ADK protein family ensures higher sequence similarity between TBY-2 ADK1 and ADK2 orthologues than between their respective homologues from Arabidopsis (Fig. 2). This led us to the conclusion that two independent gene duplications have occurred after the divergence of those species. Therefore, it can be postulated that the original ancestral ADK genes existing in Arabidopsis and tobacco were true orthologues sharing the same functions and that later both genes were duplicated after which duplicates may have acquired altered functions. The tree topology also proves that the emergence of N. tabacum (fusion of the two tobacco genomes) occurred after the gene duplication event.

Pepstats software (available on EMBL-EBI server) was used for amino acid sequence analysis. The deduced amino acid sequence of the proteins encoded by the NtADK clones predicted a PI of 4.8–4.9 and an almost identical molecular mass of ~37.5 kDa. The predicted molecular mass falls within the mass range of ADKs characterized from other species (14, 37) and is in agreement with the molecular weight of the putative tobacco ADK isolated by Laukens et al. (20).

The NtADK mRNA Level Is Modulated Significantly during the Cell Cycle—To investigate the accumulation of NtADK gene transcripts and ADK activity during the cell cycle, a TBY-2 cell suspension culture was synchronized by application of aphidicolin (21). Samples were taken immediately before (0 h) and every hour after aphidicolin removal (1–10 h). A peak of ~50% in the mitotic index was observed ~7 h after cells were released from aphidicolin block (Fig. 3B).

In expression studies, it is often difficult to distinguish homologous transcripts of duplicated genes that usually have the same length and similar or almost identical sequences. None of the known methods for investigation of gene expression in polyploidy (38) could be successfully applied for all of the NtADK genes. Due to the high degree of sequence similarity, it was impossible to specify amplify and visualize individual transcripts. Thus, we used the primers designed previously for determining the origin of the NtADK clones. As a consequence, PCR products reflected the combined expression level of two orthologues. The expression of NtADK1 (-syl and -tom type) and NtADK2 (-syl and -tom type) at the transcript level in the synchronized TBY-2 suspension-cultured cells was analyzed using semi-quantitative reverse transcription-PCR and ACTIN normalization (Fig. 3A). The levels of the NtADK1 and the NtADK2 orthologue transcripts showed a transient accumulation reaching a maximum of 5–6 h after aphidicolin release corresponding to the onset of mitosis. The transient nature of the expression of the NtADK2 orthologues appears more acute than that of the NtADK1 orthologues. This result was confirmed by a second independently performed synchronization experiment.

**TBY-2 ADK Activity Peak Corresponds with the Maximum in Mitotic Activity**—To verify that the NtADK clones encode proteins possessing ADK activity, the coding region of each clone was expressed as a fusion protein with an N-terminal His6 tag.
from either pET15b or pBAD/His vector in E. coli. Recombinant ADKs were subsequently purified on Ni\textsuperscript{2+}/H\textsubscript{11001} charged columns and analyzed by SDS-PAGE and Western blot with anti-His tag monoclonal antibody (Fig. 4, A and B). The observed migration pattern corresponds well with the predicted molecular mass of recombinant protein (i.e., 40 kDa).

For kinetic analysis, activity was measured by monitoring the formation of nucleotides from radiolabeled nucleoside substrates. The reaction conditions used (pH 7.2 and ATP:MgCl\textsubscript{2} molar ratio 4:1) were previously found to be within an optimal range for other plant ADKs (12, 14, 15, 37).

The \( K_m \) and \( V_{max}/K_m \) of TBY-2 ADK isoforms were determined for four substrates: Ado; isopentenyladenosine (iPA); zeatin riboside (ZR); and dihydrozeatin riboside (DHZR). As indicated by their \( K_m \) values, the four ADK isoforms display similar affinities for Ado and the three different types of cytokinins with the exception of ADK1T, which displays significantly (\( p < 0.05 \)) higher affinity for iPA than other cytokinins.

On the other hand, the values of \( V_{max}/K_m \) indicate that the S-type isoforms of ADK and especially ADK2S exhibited much higher reaction efficiency when cytokinins were used as substrates.

The overall ADK activity during the cell cycle was measured by monitoring the formation of AMP from radiolabeled Ado.

FIG. 1. Nucleotide and putative amino acid sequence of TBY-2 ADK. A, alignment of 76-nucleotide fragments of TBY-2 NtADK clones with corresponding fragments obtained by PCR from N. sylvestris and N. tomentosiformis. The name of each PCR fragment indicates species (Ntom, Nsyl) and the primers used for PCR (Tor1 and Tor2). Dots indicate identical residues. B, comparison of the 3' end nucleotide sequence of the short and long transcript variants of tomt-type NtADK. Identical residues are shaded. Numbers indicate the residue position. C, ClustalX multiple alignment of the amino acid sequence of four putative TBY-2 ADK isoforms. Identical residues are shaded. Heavily shaded areas indicate peptides identified by Laukens et al. (20). Sh, short; Lg, long.
The experiment was repeated with samples collected during two independent synchronization experiments. To ensure an optimal efficiency of ADK, the concentration of Ado used exceeded 4–5-fold its $K_{\text{m}}$ value. The ADK protein activity generally reflected the mRNA level. A maximum ADK activity of 26 nmol protein mg$^{-1}$ H$^{-1}$ reached at 7 h after aphidicolin release coincided perfectly with the maximum in mitotic activity (Fig. 3C).

**DISCUSSION**

Four *NtADK* Genes of TBY-2 Cell Line Encode Functional Isoforms of Adenosine Kinase—In this study, we demonstrated the presence of four *NtADK* genes in the tobacco BY-2 genome, a number that is in agreement with its ploidy level ($2n = 4x = 48$). Although it was technically impossible to confirm, by gene expression analysis, the presence of four *NtADK* transcripts separately, their isolation from the cDNA library implies that all four genes have remained functional. Bearing in mind the origin of *N. tabacum*, we determined which of the isolated genes were inherited from *N. tomentosiformis* (-tom-type genes) and from *N. sylvestris* (-syl-type genes). The alignment of *NtADK* partial nucleotide sequences with corresponding fragments obtained by PCR from the parental species revealed only very few nucleotide substitutions (99–100% identity). Sequence analysis of -tom-type *NtADK* clones revealed short and long transcript variants. Because the genomic sequence is unknown, it remains unclear whether this difference at the 3′ end is a result of either alternative polyadenylation or splicing. The 3′-untranslated region is known to contain sequences that control cytoplasmic mRNA functions including stability, translation, and localization (39–41). Tanguay and Gallie (42) have shown that increasing the length of the 3′-non-coding region of mRNA increased transcript accumulation in plants. The abundance of T-type isoforms in a TBY-2 cell has yet to be investigated.

**The Putative Role of ADK in Cytokinin Metabolism**—Recent years brought a number of findings over the function of ADK. The results of Mlejnek and Prochazka (43) and Mlejnek et al. (44) imply a participation of ADK in apoptosis. Wang et al. (45) showed that the proteins AL2 and L2, which determine pathogenicity of geminiviruses, inactivated *Arabidopsis ADK2* in vitro and after coexpression in *E. coli* and yeast. ADK activity was shown to increase after infection of *Nicotiana benthami-
ana with viruses lacking L2 and was reduced in transgenic
*N. benthamiana* expressing the viral proteins. This result strongly
supports the involvement of ADK in the plant response to the
viral infection. One possible explanation for this phenomenon
is reduced transmethylation activity observed in ADK-deficient
*Arabidopsis* plants (45, 46). In our study, we focused on
the implied yet ambiguous role of ADK in cytokinin metabolism.

Increased activity of ADK was observed during several
physiological processes (47) accompanied by rapid cell proliferation
and regulated by cytokinins such as early embryogenesis, ger-
mination and seedling growth (48), *in vitro* organogenesis (49,
50), and breaking bud dormancy (51). The ADK genes of *A. thaliana*
were found to be constitutively expressed in all of the
organs with the highest transcript level found in flowers and
roots (14). A series of experiments concerning the role of ADK
in metabolism of cytokinins seemed to confirm such an impli-
cation; however, the consistent feature of those experiments
was that the enzyme displayed higher affinity for adenine-type
substrates than for the cytokinins tested (10, 52, 53).

Comparison of the predicted tobacco ADK sequences with
sequences in the data base using Blast (27) revealed sequence
similarity with many proteins that belong to the PfkB carbo-
hydrate kinase family. The amino acid residues, present in
several plant and microbial sugar kinases and proposed to
contribute to the substrate binding site (17), are conserved in
all four deduced TBY-2 ADK polypeptide sequences. The N-
and C- terminal ends are free of deletions that were found to
affect activity and stability of mammalian ADK (54). Thus,
both *N. sylvestris*- and *N. tomentosiformis*-type sequences were
expected to encode functional ADKs.

The results of the study on the kinetic properties of the
*in vitro* expressed proteins sustained our presumption. The over-
all similar affinity of TBY-2 ADK isoforms for Ado and the
cytokinin substrates presented in this paper is in agreement
with a proposed involvement of ADK in cytokinin metabolism.
The nature and magnitude of the differences in kinetic prop-
erties of each ADK isoform reported here do not mirror the
degree of relationship of their amino acid sequence in an obvi-
ous way. Nevertheless, the differences in $V_{max}/K_{m}$ of individual
isoforms support the idea that, apart from their common role in
purine metabolism, they may fulfill new and mutually exclu-
sive metabolic functions. The ADK1T isoform displays a 10-fold
higher affinity for iPA than for the other substrates. Taking
into account a foregoing report on eventual role of ADK in
apoptosis through phosphorylation of iPA (43), one might point
at 1T isoforms as an element of apoptosis machinery. S-type
isoforms of TBY-2 ADK and especially ADK2S seem to exhibit

![Figure 3](image1.png)

**FIG. 3. Relative abundance of NtADK transcripts and ADK activity during cell cycle.** A, quantification of the two pairs of orthologues,
*NtADK1* (*syl-* and *-tom-type) and *NtADK2* (*syl-* and *-tom-type), transcript levels. mRNA accumulation is expressed as a percentage of
*NtADK1ACTIN* transcript abundance ratio at the 6-h time point. B, mitotic index of the cells immediately before (0 h) and 1-h intervals after
release from aphidicolin block. C, analysis of ADK protein level during TBY-2 cell cycle. Activity is expressed as a percentage of total ADK at the
7-h time point (26 nmol mg$^{-1}$ min$^{-1}$). Graph values represent the means ± S.E.

![Figure 4](image2.png)

**FIG. 4. Expression and purification of fusion ADK proteins from E. coli.** A, analysis of overexpressed ADK recombinant proteins
in *E. coli* by SDS-PAGE and silver staining. Lanes 1 and 10, molecular
mass marker; lanes 2, 4, 6, and 8, induced ADK1T, ADK1S, ADK2S,
and ADK2T, respectively; lanes 3, 5, 7, and 9, purified ADK1T, ADK1S,
ADK2S, and ADK2T, respectively. B, Western blot analysis with His
tag monoclonal antibody. Lane 1, molecular mass marker; lanes 3, 5, 7,
and 9, induced ADK1T, ADK1S, ADK2S, and ADK2T, respectively; and
lanes 2, 4, 6, and 8, noninduced cultures.
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TABLE II
Kinetic constants of ADK isoforms

| Isoform  | \(K_m\)  | Ado | iPA | ZR | DZR | \(V_{max}/K_m\) Ado | iPA | ZR | DZR |
|----------|----------|-----|-----|----|-----|----------------------|-----|-----|-----|
| ADKI     | 4.00 ± 1.08 | 0.30 ± 0.07 | 2.35 ± 0.04 | 3.88 ± 1.82 | 133.75 | 256.67 | 44.26 | 90.32 |
| ADKiS    | 1.88 ± 0.56 | 6.10 ± 1.56 | 2.45 ± 0.53 | 3.85 ± 1.66 | 66.67 | 340.98 | 151.02 | 262.34 |
| ADK2T    | 7.30 ± 2.39 | 8.71 ± 0.42 | 3.15 ± 1.10 | 2.55 ± 0.67 | 145.21 | 212.40 | 69.84 | 156.86 |
| ADK2S    | 3.91 ± 0.86 | 1.65 ± 0.95 | 2.20 ± 0.14 | 3.78 ± 1.15 | 237.70 | 1160.61 | 410.91 | 802.65 |

These values of \(K_m\) and \(V_{max}\) were calculated by non-linear regression with the aid of Hyper 1.1 software (Courtesy of J. S. Easterby, University of Liverpool, United Kingdom; see “Experimental Procedures”). DZR, dihydrozeatin riboside.

The values of \(K_m\) and \(V_{max}\) were calculated by non-linear regression with the aid of Hyper 1.1 software (Courtesy of J. S. Easterby, University of Liverpool, United Kingdom; see “Experimental Procedures”). DZR, dihydrozeatin riboside.

*Fig. 5. Cytokinin levels in the synchronized BY-2 cells analyzed by liquid chromatography tandem mass spectrometry. iPA, isopentenyladenine; iPMP, isopentenyladenosine monophosphate.*

Relatively higher efficiency in metabolizing cytokinin ribosides. However, the kinetic data do not allow detailed speculation about the roles of the isoforms in metabolism in vivo.

ADK Is Proposed to be Involved in the Control of the Cell Cycle Progression—To identify plant genes involved in cell division, Breyne et al. (55) performed a genome-wide expression analysis of the cell cycle-modulated genes in TBY-2 cell line. cDNA-AFLP tags, significantly altered during the cell cycle progression, comprised around 1340 genes. Among these genes, ADK exhibited a G2-M phase-specific expression pattern. The analysis of the sequence identified by Breyne et al. (55) resulted in the recognition of NtADK2-syl. Notably, this was also the only isoform reported to be induced by jasmonate treatment of tobacco BY-2 cells (56). In contrast, NtADK2-tom expression remained stable in both cDNA-AFLP analyses, whereas NtADK1-tom and NtADK1-syl gene tags were too large to allow visualization in the cDNA-AFLP experiments.5 Interestingly, ADK2S was also most likely the only isoform bound to Z in cytokinin affinity experiment performed by Laukens et al. (20) (Fig. 1C). Based on the results of the discussed experiments, it is highly probable that only –syl-type orthologues exhibit cell cycle-modulated transcript accumulation. In the present study, the expression of NtADKs genes reaches its peak in late G2, corresponding with the expression profile established by the cDNA-AFLP technique (55). One may consider the increased activity of ADK during mitosis to be associated with a high content of phosphorylated cytokinins, whereas elevated ADK activity during S phase may be linked with the metabolism of Ado coupled with the DNA synthesis. The succession of Z, ZR, and ZMP peaks prior to mitosis indicates that the latter is formed through ZR phosphorylation, rather than de novo synthesis (57, 58). Variation in the measured relative abundance of the nucleotide and nucleoside forms of Z during G2-M transition indicates interconversion between these forms (22). Our results suggest that ADK may act specifically in the cell cycle checkpoint G2-M, rapidly reducing the dramatically increased content of active cytokinins to the basal level controlling cell cycle progression. Genome-wide expression profiling using massively parallel signature sequencing allowed the identification of ADK1 gene of A. thaliana (At3g09820) among 823 genes up-regulated in response to cytokinin after the induction of bacterial isopentenyl transferase gene, IPT (59). IPT is a key enzyme in the biosynthetic pathway of cytokinins, and it has been shown that its expression in Arabidopsis is followed by significant rises in concentrations of the zeatin-type cytokinins (57). Based on its kinetic of expression, ADK1 was assigned to the group of genes that showed only a transient response to increased endogenous cytokinin levels and their expression appears to be well regulated in a closed interval (59). The role for ADK in cytokinin metabolism proposed in this paper may be additionally supported by the results of recent microarray experiments (The Nottingham Arabidopsis Stock Centre Array Data Base) (60). High expression levels of ADK1 were detected during Agrobacterium tumefaciens induced tumor development of A. thaliana. Elevated ADK transcript accumulation in the tumor tissue correlates with excessive production of Z and ZR observed previously by Azmi et al. (61), whereas Veselov et al. (62) reported on a large cytokinin nucleotide pool increasing steadily throughout the growth period of the tumor.

The data presented in this paper contribute to our understanding of cytokinin metabolism underlying the cell cycle. Correlation among ADK expression, activity, in vitro properties, and ZMP accumulation implies a role for ADK during tobacco BY-2 cell cycle progression. A similar role was proposed for cytokinin oxidase/dehydrogenase or CKX protein (63). Expression of CKX genes in zones of active cell division in Arabidopsis is in agreement with the anticipated function in degragation of cell cycle-derived cytokinins; however, it cannot explain the appearance of a transient ZMP peak. Hence, it is

5 A. Goossens, unpublished data.
safe to presume that several different enzymatic activities are involved in cytokinin metabolism at this stage of the cell cycle. The results of this study particularly point at ADR2S as the isoform potentially involved in the cell cycle-regulated metabolism of cytokinins. Nevertheless, the nature of this work remains strictly correlative and much more needs to be done to confirm the role of the NtADK genes in the cytokinin metabolism in vivo as well as the link between the regulation of the NtADK genes expression and the regulation of the cell cycle. To complete the functional analysis and to answer the question of whether different isoforms fulfill different metabolic functions within a plant cell, more elaborated research on promoter activity and the effect of selective RNAi silencing is currently being conducted in our group.

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