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SHORT COMMUNICATION

Cloning and characterization of an adenosine kinase from *Physcomitrella* involved in cytokinin metabolism

Klaus von Schwartzenberg[1,*], Sven Kruse[2], Ralf Reski[2], Barbara Moffatt[3] and Michel Laloue[1]

[1]*Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique Centre Versailles, F-78026 Versailles Cedex, France,*

[2]*Institut Biologie II, Albert-Ludwigs-Universität Freiburg, Schänzlestr. 1, D-79104 Freiburg, Germany, and*

[3]*Biology Department, University of Waterloo, ON, Canada N2L 3G1*

Summary

Adenosine kinase (*adk*) from the moss *Physcomitrella patens* (Hedw.) B.S.G. was cloned from a cDNA library by functional complementation of an *Escherichia coli* purine auxotrophic strain. The length of the entire cDNA clone was 1175 bp with an open reading frame coding for a protein with a predicted molecular weight of 37.3 kDa. Southern analysis indicated the presence of a single adenosine kinase gene within the *Physcomitrella* genome. The deduced amino acid sequence had a 52% identity with the human adenosine kinase. The transfer of phosphate from ATP to adenosine resulting in AMP, as well as the phosphorylation of the cytokinin, isopentenyladenosine, to isopentenyladenosine monophosphate, was shown by *in vitro* enzyme assays using crude extracts from *E. coli* mutants expressing the *adk* cDNA clone and from *Physcomitrella* chloronemal tissue. Results from *in vivo* feeding of chloronemal tissue with tritiated isopentenyladenosine suggest that adenosine kinase plays an important role in the conversion of cytokinins towards their nucleotides in *Physcomitrella*.

Introduction

Adenylic compounds can be interconverted between the base, the riboside and the nucleotide form via the enzymes of the adenine salvage pathway. Recycling of adenosine into the nucleotide AMP can occur by two different routes (Figure 1). One is a two-step reaction using adenosine nucleosidase or purine nucleoside phosphorylase to form adenine and a subsequent ribophosphorylation by adenine phosphoribosyltransferase (APT). The second pathway is the direct phosphorylation of adenosine by adenosine kinase (ADK, E.C.2.7.1.20).

There is evidence that the purine plant hormones, cytokinins, can also serve as substrates for adenine salvage enzymes *in vitro* (Chen and Eckert, 1977; Chen *et al.*, 1982; Doree and Terrine, 1973; Lee and Moffatt, 1994; Mok and Martin, 1994; Schnorr *et al.*, 1996). Furthermore, the formation of cytokinin nucleotides after feeding plant tissue with a radiolabelled cytokinin base or riboside is a major metabolic step in cytokinin metabolism. Nucleotides, as low active forms of cytokinins, are dominant metabolic products in the early stages following exogenous cytokinin application (Auer *et al.*, 1992; reviewed in McGaw, 1995).

For the adenine phosphoribosyltransferase pathway in *Arabidopsis thaliana*, two different genes have been cloned and described (Moffatt *et al.*, 1992; Schnorr *et al.*, 1996). AT*apt*1 has a 166-fold higher K_m for the cytokinin benzyladenine compared to adenine (Schnorr *et al.*, 1996); however, a mutation of At*apt*1 in *A. thaliana* leads to a strongly reduced ribophosphorylation of benzyladenine *in vivo* and *in vitro* (Moffatt *et al.*, 1991). AT*apt*2 has a K_m for benzyladenine threefold lower than that for adenine and its affinity for benzyladenine is 166-fold higher than that of AT*apt*1 to benzyladenine. A similar situation exists in tomato, which has leaf and root forms of APT that differ in their relative affinities for cytokinin and adenine (Burch and Stuchbury, 1986).

Concerning purine interconversion there is so far no report on an enzyme with absolute specificity for cytokinins. However, the elevated relative specificity of AT*apt*2 for benzyladenine suggests that it may have a major role in the formation of cytokinin nucleotides in *A. thaliana*. Recently, a third form of *apt* has been identified in the

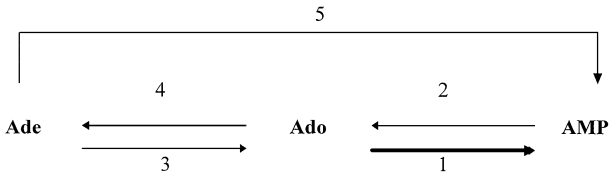

Figure 1. Enzymes involved in purine interconversion in higher plants.
1 – adenosine kinase; 2 – 5′ nucleotidase; 3 – purine nucleoside phosphorylase; 4 – adenosine nucleosidase; 5 – adenine phosphoribosyltransferase. Ade – adenine; Ado – adenosine; AMP – adenosine 5′-monophosphate. The enzymes mentioned are also reported to be cytokinin metabolic enzymes (Mok and Martin, 1994).

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*For correspondence: Institut für Angewandte Botanik, Universität Hamburg, Marseiller Str. 7, D-20355 Hamburg, Germany (fax +49 40 41236593; e-mail kvschwartzenberg@iangbot.uni-hamburg.de).

A. thaliana EST collection (R305577; B. Moffatt *et al.*, unpublished results). The enzymatic specificity of this APT has yet to be determined.

The enzyme activities involved in the re-use of adenosine have been found in several plant tissues (Chen and Eckert, 1977; Guranowski, 1979; Guranowski and Wasternack, 1982; Le Floc'h and Faye, 1995). There is also a report from an overall study of purine metabolism in *Nicotiana tabacum* protoplasts that did not detect ADK activity (Barankiewicz and Paszkowski, 1980). The affinity of a semi-purified preparation of wheat germ adenosine kinase for the cytokinin riboside isopentenyladenosine is reported to be about fourfold lower compared to its affinity for adenosine (Chen and Eckert, 1977; Chen, 1982).

The first cDNA for adenosine kinase was recently isolated from *Homo sapiens sapiens* after enzyme

(a)

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CGCGCTAGATCCCTCGCTCTTATTCGACCACGCATTCTCTCTCTCGCAATGGCGTCCGAGGGTGTGCTTTTG      72
                                                  M  A  S  E  G  V  L  L       8

GGCATGGGAAACCCCCTGCTCGACATCTCCTGCGTGGTCGACGACGCATTCCTCGAGAAGTACGGGCTGACG     144
 G  M  G  N  P  L  L  D  I  S  C  V  V  D  D  A  F  L  E  K  Y  G  L  T      32

CTAAACAACGCTATTCTTGCTGAGGACAAGCACCTTCCCATGTACAAGGAACTGGCTGCCAATCCCGATGTA     216
 L  N  N  A  I  L  A  E  D  K  H  L  P  M  Y  K  E  L  A  A  N  P  D  V      56

GAGTACATTGCAGGAGGTGCTACTCAGAACACCATCAGGATTGCCCAGTGGATGCTAGGTGAATCGAACGCA     288
 E  Y  I  A  G  G  A  T  Q  N  T  I  R  I  A  Q  W  M  L  G  E  S  N  A      80

ACTAGCTACTTTGGCTGTGTTGGCAAGGATGAGTATGGCGACCGTATGTTCAAGCTCGCCTCTGAGGGAGGT     360
 T  S  Y  F  G  C  V  G  K  D  E  Y  G  D  R  M  F  K  L  A  S  E  G  G      104

GTCAATATCCGATACGATGTGGACGAGGATCTTCCCACTGGAACATGCGGCGTGCTCGTGGTGAAGGGAGAG     432
 V  N  I  R  Y  D  V  D  E  D  L  P  T  G  T  C  G  V  L  V  V  K  G  E      128

AGGTCCTTGGTAGCCAATCTTTCAGCCGCCAACAAATACAAGATCGACCACTTGAAGAAGCCAGAAAACTGG     504
 R  S  L  V  A  N  L  S  A  A  N  K  Y  K  I  D  H  L  K  K  P  E  N  W      152

GCTTTCGTGGAGAAGGCAAAGTACATCTACAGCGCCGGTTTCTTCCTGACTGTTTCACCGGAATCTATGATG     576
 A  F  V  E  K  A  K  Y  I  Y  S  A  G  F  F  L  T  V  S  P  E  S  M  M      176

ACCGTGGCCAAACATGCTGCCGAGACCGGAAAATACTACATGATCAACTTAGCCGCTCCGTTCATCTGCCAG     648
 T  V  A  K  H  A  A  E  T  G  K  Y  Y  M  I  N  L  A  A  P  F  I  C  Q      200

TTCTTTAAGGACCCTCTTATGGAGCTTTTCCCTTACGTGGATTTCATTTTCGGCAACGAGAGCGAGGCCAGA     720
 F  F  K  D  P  L  M  E  L  F  P  Y  V  D  F  I  F  G  N  E  S  E  A  R      224

GCATTTGCGCAAGTTCAAGGCTGGGAGACAGAGGACACCAAGGTGATAGCCGTAAAGTTGGCTGCGTTACCG     792
 A  F  A  Q  V  Q  G  W  E  T  E  D  T  K  V  I  A  V  K  L  A  A  L  P      248

AAAGCTGGCGGCACCCACAAGCGTGTCGCTGTCATCACCCAGGGAACTGACCCCACAATTGTTGCTGAAGAT     864
 K  A  G  G  T  H  K  R  V  A  V  I  T  Q  G  T  D  P  T  I  V  A  E  D      272

GGAAAGGTGACTGAATTCCCCGTCACCCCTATTCCTAAGGAGAAGTTGGTCGACACTAATGCAGCTGGTGAC     936
 G  K  V  T  E  F  P  V  T  P  I  P  K  E  K  L  V  D  T  N  A  A  G  D      296

TCTTTTGTCGGAGGGTTCTTGTCTCAGCTGGTGTTGGGTAAAGACATCGCACAGTGCGTCAGAGCAGGAAAC    1008
 S  F  V  G  G  F  L  S  Q  L  V  L  G  K  D  I  A  Q  C  V  R  A  G  N      320

TACGCAGCCAGCGTCATCATCCAGCGCTCTGGATGCACTTTCCCTTCCAAACCATCCTTCGAAAGTCAGTAG    1080
 Y  A  A  S  V  I  I  Q  R  S  G  C  T  F  P  S  K  P  S  F  E  S  Q  *      344

AAAATTGTTAGGGTAGGGGTGAGGAAATTTGCCGCCCCTTTTTTTTGTCACAGCATCTATCATGCAATACAA    1152

CTGGGTTGCCATATCAAAAAAAA                                                      1175
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Figure 2.

purification, peptide sequencing and screening of a cDNA library (Spychala *et al.*, 1996). Recently EST clones with homology to human adenosine kinase have been identified in the *Arabidopsis* database and the corresponding genes have been isolated (B. Moffatt, L. Wang and K. von Schwartzenberg, unpublished results). So far, no plant gene encoding adenosine kinase has been described.

In this paper we report the isolation and characterization of a plant adenosine kinase cDNA from the moss *Physcomitrella patens* (Hedw.) B.S.G. by functional complementation of a purine auxotrophic *Escherichia coli* mutant and a characterization of its activity on both adenosine and cytokinin substrates.

Results and discussion

Isolation and characterization of adk *cDNA*

Functional complementation relied on a cDNA library from *Physcomitrella* prepared in λ-ZAPII (Reski *et al.*, 1995). The

(b)

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P. patens     MAS--EGVLLGMGNPLLDISCVVDDAFLEKYGLTLNNAILAEDKHLPMYKELAANPDVEY   58
Human         MTSVRENILFGMGNPLLDISAVVDKDFLDKYSLKPNDQILAEDKHKELFDELVKKFKVEY   60
Rat           -----------MGNPLLDISAVVDKDFLDKYSLKPNDQILAEDKHKELFDELVKKFKVEY   49
Hamster       -----------MGNPLLDISAVVDKDFLDKYSLKPNDQILAEEKHKELFDELVRKFKVEY   49
                        *********.***  **.** *  *  ****.**  .. **     ***

                                                  motif 1
P. patens     IAGGATQNTIRIAQWMLGE-SNATSYFGCVGKDEYGDRMFKLASEGGVNIRYDVDEDLPT   117
Human         HAGGSTQNSIKVAQWMIQQPHKAATFFGCIGIDKFGEILKRKAAEAHVDAHYYEQNEQPT   120
Rat           HAGGSTQNSMKVAQWMIQEPHRAATFFGCIGIDKFGEILKSKAANAHVDAHYYEQNEQPT   109
Hamster       HAGGSTQNSIKVAQWMIQKPHKAATFFGCIGIDKFGEILKSKAAEAHVDAHYYEQNEQPT   109
               ***.***....****.     *...***.* * .*. .   *.   *  .*    . **

P. patens     GTCGVLVVKGERSLVANLSAANKYKID-HLKKPENWAFVEKAKYIYSAGFFLTVSPESMM   176
Human         GTCAACITGDNRSLIANLAAANCYKKEKHLDLEKNWMLVEKARVCYIAGFFLHVSPESVL   180
Rat           GTCAACISGGNRSLVANLRAANCYKKEKHLDLENNWMLVEKARVYYIAGFFLTVSPESVL   169
Hamster       GTCAACITGDNRSLVANLAAANCYKKEKHLDLENNWVLVEKARVYYIAGFFLTVSPESVL   169
              ***   .    ***.*** *** ** . **    **  ****.  * ***** *****..

P. patens     TVAKHAAETGKYYMINLAAPFICQFFKDPLMELFPYVDFIFGNESEARAFAQVQGWETED   236
Human         KVAHHASENNRIFTLNLSAPFISQFYKESLMKVMPYVDILFGNETEAATFAREQGFETKD   240
Rat           KMARYAAENNRTFTLNLSAPFISQFFKEALMAVMPYVDILFGNETEAATFAREQGFETKD   229
Hamster       KVARYAAENNRIFTLNLSAPFISQFFKESLMEVMPYVDILFGNETEAATFAREQGFETKD   229
               .*. *.*... . .**.****.**.*. ** . **** .****.** .**. ** ** *

                                                                 motif 2
P. patens     TKVIAVKLAALPKAGGTHKRVAVITQGTDPTIVAEDGKVTEFPVTPIPKEKLVDTNAAGD   296
Human         IKEIAKKTQALPKMNSKRQRIVIFTQGRDDTIMATESEVTAFAVLDQDQKEIIDTNGAGD   300
Rat           IKEIADKTQALPKVNSKRQRTVIFTQGRDDTIVATGNDVTAFPVLDQNQEEIVDTNGAGD   289
Hamster       IKEIAKKAQALAKVNSKRPRTVVFTQGRDDTVVATENEVMAFAVLDQNQKEIIDTNGAGD   289
               * ** *  ** * .  . *  . *** * *..*    *  * *    .  ..*** ***

P. patens     SFVGGFLSQLVLGKDIAQCVRAGNYAASVIIQRSGCTFPSKPSFESQ                343
Human         AFVGGFLSQLVSDKPLTECIRAGHYAASIIIRRTGCTFPEKPDFH--                345
Rat           AFVGGFLSQLVSNKPLTECIRAGHYAASVIIRRTGCTFPEKPDFH--                334
Hamster       AFVGGFLSQLVYNKPLTECIRAGHYAASVIIRRTGCTFPEKPDFH--                334
              .**********  * ...*.***.****.**.*.***** ** *
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Figure 2. (a) Nucleotide and amino acid sequences of adenosine kinase cDNA of *Physcomitrella patens.* The amino acid sequence was translated starting from the first in- frame ATG.
(b) The amino acid sequence of *P. patens adk* cDNA was aligned with the human *adk* (Spychala *et al.*, 1996; accession no. U50196), hamster *adk* (*Cricetulus griseus*; R.S. Gupta, B. Singh and W. Hao, unpuplished data; Genbank accession no. U26588) and rat *adk* (*Rattus rattus*; R.S. Gupta, W. Hao, Z.-C. Wu and B. Singh, unpublished data; accession no. U57042) using the Clustal W program (Thompson *et al.*, 1994). * Identical amino acids; '.', similar amino acids. Letters in italic refer to motifs 1 and 2 described by Spychala *et al.* (1996).

cDNA library was *in vivo* excised into a pBluescript plasmid library using the helper phage M13. Double-stranded plasmid DNA was prepared and electroporated into the *E. coli* purine-deficient mutant HO4 (Hove-Jensen and Nygaard, 1989).

The HO4 strain carries several mutations, four of which are purine mutations relevant to this study: *purE*, *purM*, *deoD* and *apt*. Two mutations (*purE* and *purM*) affect the *de novo* purine biosynthetic pathway, thereby making the strain strictly purine auxotrophic. The mutation of the *deoD* locus is important to protect the chosen purine substrate adenosine from degradation to adenine by purine nucleoside phosphorylase. In spite of these mutations in HO4, there remains a slight degradation of adenosine to adenine, which is due to the 5′-methylthioadenosine phosphorylase (Nygaard, 1983), although this cannot result in growth in the absence of complementation as the *apt* locus encoding for adenine salvage enzyme APT is also inactivated. Furthermore, *E. coli* does not have adenosine kinase (Nygaard, 1983). A selective medium was designed to allow only those bacteria to grow that have received a cDNA conferring the ability to phosphorylate adenosine. The AMP generated could be metabolized further by the histidine biosynthetic pathway to inosine monophosphate and guanylates. The selection medium contained deoxicoformycin to inhibit adenosine deaminase activity, thereby preventing the supplied adenosine from being degraded to inosine. Under the conditions described *apt* cDNA clones can also be selected (Schnorr *et al.*, 1996). In order to reduce the occurence of *apt* complemented colonies, the adenine analogue 2-fluoroadenine was added, which when ribophosphorylated by APT gives a highly toxic nucleotide.

After electroporation of the cDNA library into HO4 the overall transformation rate was 8×10^7 clones per μg DNA. Six clones were isolated after plating cells on selective medium (containing adenosine, ampicillin, deoxicoformycine and 2-fluoroadenine). Restriction analysis of the cDNA inserts from the purified colonies suggested that, despite 2-fluoroadenine counter-selection, three of the clones corresponded to *apt*, which had been cloned from *Physcomitrella* in a separate study by a similar method (K. von Schwartzenberg and M. Laloue, unpublished data). Sequencing of these clones verified this conclusion.

The plasmid DNA of two of the remaining clones was isolated and re-transformed into HO4. The growth of these transformants indicated that the capability to grow on selective medium resided on the plasmid. Sequencing of the 3′ and 5′ ends of the clones indicated a strong homology of the inserts with the human *adk* cDNA (Spychala *et al.*, 1996). One clone (pPp*adk*) was selected to generate subclones to facilitate the determination of its entire nucleotide sequence (Figure 2). The predicted molecular weight of the largest open reading frame starting at the first methionine (343 amino acids) is 37 254 Da. The cDNA clone contains 5′ and 3′ non-translated regions of 48 and 95 nucleotides, respectively (Figure 2a).

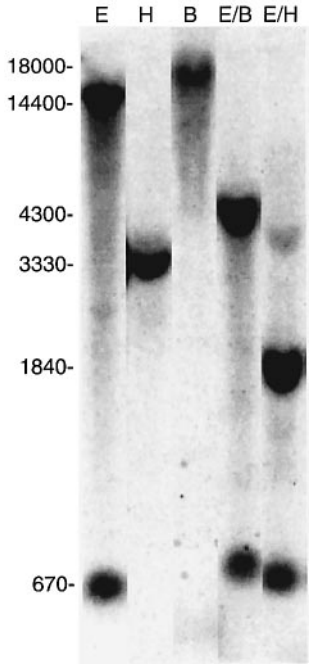

Figure 3. Southern analysis of genomic *P. patens* DNA.
Probe: large *Xho*I-fragment of Pp*adk*. Digests: E – *Eco*RI; H – *Hind*III; B – *Bam*HI; E/B – *Eco*RI/*Bam*HI; E/H – *Eco*RI/*Hind*III. Numbers indicate fragment size (nucleotides).

Southern and Northern analysis

The restriction pattern of *Eco*RI-, *Hin*dIII- and *Bam*HI-digested genomic DNA hybridized with the *adk* probe under high stringency conditions confirms the presence of the *adk* gene in the genome of *Physcomitrella.* One band is labelled for the *Hind*III- and *Bam*HI-digested DNA, and two bands are labelled for the *Eco*RI-digested DNA. This is consistent with the restriction analysis of the *adk* cDNA that contains an internal *Eco*RI site. Thus the data suggest that *adk* is a single copy gene in *Physcomitrella* (Figure 3).

The mRNA size for *adk* mRNA as determined by Northern hybridization was found to be 1400 nt (data not shown). It is likely that the *adk* cDNA, with a length of 1175 nt, lacks a part of its 5′-untranslated region.

Function of the adenosine kinase cDNA

Growth tests. Biological evidence for the formation of AMP by the *Physcomitrella adk* cDNA was obtained from growth assays using the purine auxotrophic mutant HO4 transformed with the *adk* cDNA in pBluescript. On selective medium (see Functional complementation in the Experimental procedures) containing 10 μg ml^{-1} histidine, the growth of the colonies was significantly inhibited in number

and size in comparison to the growth on medium without histidine (data not shown).

This result suggested that under non-restrictive conditions the AMP is further phosphorylated to ATP and then transformed into AICAR (aminoimidazolcarbamidribonucleotide), a byproduct in the histidine biosynthetic pathway. In a purine auxotrophic background, such as HO4, the AICAR formed in the histidine biosynthesis pathway serves as the only source of guanylates.

For enteric bacteria it is known that the first enzyme of the histidine biosynthetic pathway is feedback-inhibited by histidine (Nygaard, 1983). It is likely that the histidine-mediated growth inhibition of *adk*-complemented HO4 is related to the repression of the histidine biosynthetic pathway depriving cells of guanylates.

Sequence comparison of Physcomitrella *ADK with other ADK*

The deduced amino acid sequence of *Physcomitrella adk* cDNA shows a 52% identity and 67% similarity to the human adenosine kinase recently published by Spychala *et al.* (1996). When compared to other *adk* GenBank entries. the highest identity score of 55% was obtained with the rat (*Rattus rattus*) clone.

A relatively low homology of 15% was found with respect to a ADK sequence of the trypanosome *Leishmania donovani* (P. Misra, D. Bandyopadhyay and A.K. Datta, unpublished data; GenBank accession no. Z35297, data not shown). When aligning the mammalian *adk* amino acid sequences with each other, they exhibit identities of more than 90%, whereas homologies with the *L. donovani* clone are also low with identity scores of 14–15%. Obviously, the *L. donovani* sequence is clearly separated in the phylogeny of the known *adk* genes.

Spychala *et al.* (1996) describe two motifs within the human *adk* gene that are also clearly conserved in the plant *adk* (Figure 2b). Motif 1 (from residue 86–109) is shared with microbial ribokinases, fructokinases and fructokinase from potato but not with inosine kinase and 1-phosphofructokinase from *E. coli*. Motif 2 however, is found in all fructokinases, including 1-phosphofructokinase from *E. coli* as well as inosine kinase (*E. coli*) and alpha-amylase from yeast. As the substrates for those enzymes carry a furanose moiety, Spychala *et al.* (1996) suggest that motif 2 in *adk* is involved in binding of the pentose moiety; this hypothesis can also be put forward for *Physcomitrella adk*.

Enzyme assays and in vivo *feeding experiments*

The *adk* cDNA was expressed in *E. coli* HO4 grown in LB medium in the presence of isopropyl thiogalactopyranoside (IPTG). Subsequently protein extracts were prepared and *in vitro* enzyme assays were performed using radiolabelled purine substrates and ATP as the phosphate donor. The enzyme assay was based on the precipitation of the purine nucleotides formed with lanthanium chloride and subsequent liquid scintillation counting.

It was shown that the gene product of the *adk* cDNA was able to catalyse the expected phosphorylation of ^{14}C-labelled adenosine. The measured activity for the IPTG-induced strain was ninefold above the background values measured for the extract of cells carrying the pBluescript vector as a control. Nucleotide formation was dependent on the presence of the co-substrate ATP.

Adding IPTG to the *E. coli* culture in order to derepress *lac* promoter activity caused ADK activity to increase threefold over the uninduced strain and ninefold over the same host carrying the vector alone (Table 1).

HPLC separation of the assay mixture after incubation confirmed the formation of adenylic nucleotides. These analyses revealed that the AMP was further phosphorylated to ATP during the assay (data not shown).

ADK activity was also detected in extracts of *Physcomitrella* chloronemal tissue (Table 1).

Using the tritiated cytokinin isopentenyladenosine ([9R]iP) as a substrate, we were able to measure the formation of isopentenyladenosine monophosphate ([9R-MP]iP) in recombinant *E. coli* extracts as well as in *Physcomitrella* chloronema extracts (Table 2). As low concentrations of substrate (85 nM) were used, the measured activities were also low. However, activities were clearly measurable because ^{3}H-[9R]iP with a high specific radioactivity of 666 Gbq mmol^{-1} (Laloue and Fox, 1987) was used. The reaction product co-chromatographed with [9R-MP]iP in HPLC (anion-exchange and reverse phase chromatography). A further phosphorylation to di- and triphosphates such as for the assay with adenosine was not found (data not shown).

A detailed enzymological investigation on *Physcomitrella* ADK is planned in order to determine its specificity towards adenosine and cytokinin substrates.

For *Physcomitrella*, isopentenyladenine is reported to be the main cytokinin (Wang *et al.*, 1981). In the case of the cytokinin overproducing *ove*-mutants, this compound can be found in large quantities in the culture medium (Wang *et al.*, 1980). When applying a combined HPLC enzyme-immunoassay technique we also detected isopentenyladenosine as a native cytokinin in the tissue and the culture medium of *Physcomitrella* wild type (P. Schulz, K. von Schwartzenberg and M. Laloue, unpublished data).

In order to investigate how far isopentenyladenosine could be converted *in planta* by ADK to isopentenyladenosine monophosphate, we carried out *in vivo* feeding studies with tritiated isopentenyladenosine. The results in Table 3 show that after 45 and 90 min of feeding, 86% and 94% of the radiaoactivity in the chloronemal extract was already bound to the fraction of the cytokinin nucleotides. When

Table 1. *In vitro* enzyme activity for adenosine kinase towards ^{14}C-adenosine (16 μM) in extracts from *E. coli* (*E.c.*) HO4 mutant and *Physcomitrella* (*P.p.*) wild-type (wt) chloronema tissue (SD given in parentheses, $n = 3$). pBSSK – Bluescript control (vector); pPp*adk* – *Physcomitrella* adenosine kinase cDNA in pBSSK. The protein concentration of desalted crude extracts was 10–50 μg per assay (vol. 50 μl). Control assays lacking extract resulted in no measurable activity

			Activity	pmol
Extract strain/plasmid	IPTG induction (*E. coli* strains)	ATP (5 mM)		min mg protein
E.c. HO4/pBSSK control	+	+	50 (± 5.1)	
E.c. HO4/pPp*adk*	+	+	450 (± 113)	
E.c. HO4/pPp*adk*	–	+	145 (± 37.7)	
E.c. HO4/pPp*adk*	+	–	18 (± 0.66)	
P. p. wt chloronema		+	582 (± 162)	
P.p. wt chloronema		–	7 (± 2.5)	

Table 2. Adenosine kinase activity towards tritiated isopentenyladenosine (85 nM) in *E. coli* (*E.c.*) extracts containing *adk* cDNA and *Physcomitrella* (*P.p.*) chloronema tissue. The protein concentration was 10 μg per incubation. ATP, when added to the assay, was 5 mM. Control assays lacking extract resulted in no measurable activity. [9R-MP]iP was quantified by RP-HPLC and liquid scintillation counting

		Activity	pmol
Extract	ATP		min mg protein
E.c. HO4/pPp*adk*	+	0.5	
E.c. HO4/pPp*adk*	–	*	
P.p. wt chloronema	+	0.13	
P.p. wt chloronema	–	*	

*Under detection limit of 0.005 pmol min^{-1} mg $protein^{-1}$.

Table 3. Cytokinin nucleotide formation during *in vivo* feeding of *Physcomitrella* chloronema cells in tritiated isopentenyladenosine [9R]iP (0.3 μM). Data present radioactivity in percentage of total radioactivity in extract

Tritiated metabolites	45 min feeding	90 min feeding
[9R]iP	14	3
iP	Not detected	3
[9R]iP – nucleotides	86*	94**

*Only [9R]iP monophosphate.
**Includes mono-, di- and triphosphates of [9R]iP.

using the cytokinin base (tritiated isopentenyladenine) for feeding, no significant incorporation of radioactivity into the fraction of cytokinin nucleotides was found (data not shown). These results suggest that in *Physcomitrella* the ADK pathway – and not the APT pathway – is involved in the conversion of cytokinins towards their nucleotides.

A detailed analysis of the relative specificity of ADK towards adenosine and cytokinin ribosides, as well as studies concerning the expression of adenosine kinase, should help us to understand how far enzymes from purine/cytokinin interconversion are involved in the regulation of cytokinin activity in this organism.

Experimental procedures

Plant material and growth conditions for cDNA library preparation

Physcomitrella patens (Hedw.) B.S.G. wild type has been described previously (Reski *et al.*, 1994). Plants were axenically grown under standard conditions (agitated liquid medium, 1.84 mM KH_2PO_4, 1.01 mM $MgSO_4$, 3.35 mM KCl, 4.23 mM Ca $(NO_3)_2$, 0.045 mM $FeSO_4$, pH 5.8) in a growth chamber under controlled conditions (25 ± 1°C; light provided from above by two fluorescent tubes Philips TL 65 W 25^{-1}; light flux of 55 μmol s^{-1} m^{-2} outside the flasks, light–dark regime of 16:8 h). Nine-day-old protonemata were used for preparation of RNA.

Growth conditions for enzyme extract preparation and in vivo *feeding*

For the production of chloronemal filaments, the moss was cultivated in liquid culture using a medium according to Wang *et al.* (1980): $Ca(NO_3)_2$ 0.36 mM, $FeSO_4$ 0.035 mM, $MgSO_4$ 1.01 mM, KH_2PO_4 1.84 mM, KNO_3 10 mM; 1 ml of Hoagland's trace element solution was added (Ashton and Cove, 1977). NH_4-tartrate, 5 mM, was added and the pH was adjusted to 6.5. The culture (500 ml) was aerated with water-saturated sterile air (⊤ 600 ml min^{-1}). A seven-day-old culture consisting of chloronemal filaments was used for enzyme extract preparation and *in vivo* feeding studies with tritiated cytokinins.

cDNA library preparation

Five micrograms of polyadenylated RNA was used to construct a cDNA library (cDNA-Synthesis-Kit, Stratagene Inc., La Jolla, CA) in λ ZAP II (Reski *et al.*, 1991; Reski *et al.*, 1995).

The λ ZAP II cDNA library was *in vivo* bulk excised according to Short *et al.* (1988), resulting in a phagemid library. After infection

of *E. coli* XL1-Blue and plating on large Petri dishes, a double-stranded plasmid cDNA library was isolated from ampicillin-resistant colonies using a plasmid Qiagen-midiprep procedure (Qiagen Inc., Chatsworth, CA).

Bacterial strains

Escherichia coli HO4 *metB*, *rspL*, *relA*, *spoT*, *supF*, *lamB*, *purE*, *deoD*, *apt*, *purM:: Mucts62*, *gsk-9* (Hove-Jensen and Nygaard, 1989) was obtained courtesy of P. Nygaard (Institute of Biological Chemistry, Copenhagen, Denmark).

XL1-Blue *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [F′ proAB, lacI^q, ZΔ, M15, Tn10] was obtained from Stratagene Inc.

Functional complementation

Electrocompetent *E. coli* HO4 cells were prepared according to the method of Dower *et al.* (1988). An aliquot of 40 μl of electrocompetent cells was electroporated with 300 ng of double-stranded cDNA library using a Bio-Rad Gene Pulser set at 12.5 kV cm^{-1}, 200 μF capacity and 200 Ohm shunt resistance. The cells were resuspended in 1 ml of LB medium and incubated for 1.5 h on a rotary shaker (200 r.p.m.) at 28°C in a sterile tube. The suspension was centrifuged (3000 ***g***, 5 min) and the cells were resuspended in 200 μl of 10 mM $MgSO_4$.

Cell suspension, 100 μl, was plated on M9 medium (Sambrook *et al.*, 1989) containing 1 mM IPTG, 100 μg ml^{-1} ampicillin, 10 μg ml^{-1} methionine, 1 μg ml^{-1} thiamine, 0.2% mannitol and 100 μM adenosine. The medium was also supplemented with 0.1 μM 2-deoxicoformycin and 2 μM 2-fluoroadenine. These latter substances were obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). For selection of complemented *E. coli*, the Petri dishes were incubated at 28°C for 3 days. Colonies were streaked out on new selective medium and incubated further. Plasmid DNA was isolated according to Del Sal *et al.* (1989) and again electroporated into *E. coli* HO4 cells as described above in order to confirm plasmid-borne complementation.

DNA sequencing

The pBluescript SK ± vector containing the adenosine kinase cDNA was amplified in *E. coli* XL1-Blue, and plasmid was isolated by ion-exchange chromatography (Qiagen Inc.). Several unidirectional deletions from the 3′- and the 5′-end of the cDNA insert were prepared by exonuclease III treatment (Sambrook *et al.*, 1989). The nucleotide sequence of the resulting subclones was determined in both directions using a modified dideoxy-terminator method (Sanger *et al.*, 1977). Sequencing was performed on an Applied Biosystems model 373 automated sequencer using the PRISM™ reagents (Applied Biosystems, Foster City, CA). Sequence data were analysed by GeneJockey software (Biosoft Inc, Cambridge, UK). Alignments of amino acid sequences were made using CLUSTAL W (Thompson *et al.*, 1994).

Isolation of genomic DNA and Southern analysis

Isolation of genomic DNA and subsequent Southern analyses were carried out as described previously (Reski *et al.*, 1991) from material grown in large-scale cultures as described above. Three micrograms of DNA were utilized for each restriction digest. The cDNA probe was labelled with the DIG random priming labelling kit (Boehringer Mannheim, Mannheim, Germany).

For high stringency hybridization the filters were incubated at 42°C for 3 h in presoak consisting of 0.2 g glycin, 3.2 ml deionized water, 5 ml 20 × SSPE [20 × SSPE: 20 mM EDTA, 200 mM NaH_2PO_4, 3.6 M NaCl, 210 mM NaOH (pH 7)], 2 mg salmon sperm DNA, 1.0 ml 100 × Denhardt's solution [100 × Denhardt: 2% bovine serum albumin (BSA), 2% Ficoll, 2% polyvinylpyrolidone], 10 ml formamide and 60 mg sodium dodecyl sulphate (SDS) per 20 ml. Hybridizations were carried out in hybridization solution (i.e. preasoak without glycine and supplemented with 2.0 g dextrane sulphate and the probe) for 16 h at 42°C. Filters were washed twice in 2 × SSPE, 0.3% SDS and twice in 0.1 × SSPE, 0.3% SDS for 15 min, each step at 42°C.

Preparation of enzyme extracts from E. coli *expressing ADK*

An *E. coli* HO4 secondary transformant containing the adenosine kinase cDNA in the *Eco*RI site of pBluescript SK, as well as HO4 containing pBluescript SK (wild type, control), was grown overnight in liquid LB medium supplemented with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cultures (250 ml) were centrifuged and the bacterial pellet was frozen at – 20°C. Cells were resuspended in 10 ml of extraction buffer (50 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$) and 10 mg of lysozyme was added. After incubation for 40 min at room temperature, the mixture was sonicated for 2 min. DNase I (Boehringer Mannheim) was added to a final concentration of 10 μg ml^{-1}. After incubation for 30 min at room temperature, the mixture was centrifuged at 10 000 ***g*** (20 min, 4°C). Solid NH_4SO_4 was added to the supernatant to saturation and the mixture was incubated for 45 min on ice. After centrifugation at 10 000 ***g*** for 35 min, the pellet was resuspended in 10 ml of extraction buffer. The solution was again centrifuged and desalted on NAP10 columns (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Protein concentration of the desalted extract was measured according to Bradford (1976) and adjusted to 5 mg ml^{-1}. Extracts were stored at – 20°C.

Preparation of enzyme extracts from Physcomitrella

Ten grams fresh weight of *Physcomitrella* chloronema tissue was ground in liquid nitrogen with mortar and pestle to a fine powder. Twenty millilitres of extraction buffer (Tris–HCl 50 mM, pH 7.5, DTT 2 mM, EDTA 5 mM) was added and further homogenized for 5 min at 1–2°C. The mixture was centrifuged at 10 000 ***g*** (20 min, 4°C) and extract preparation was continued as described above.

Enzyme assays

Adenosine kinase activities in extracts of recombinant *E. coli* HO4 or *Physcomitrella* were estimated by measuring the formation of 5′-ribonucleotides during the incubation with ^{14}C-adenosine or ^{3}H-isopentenyladenosine. The assay mixture was: Tris–HCl 50 mM, 1 mM $MgCl_2$, 0.1 mg ml^{-1} BSA, 15 mM NaF and 5 mM ATP (pH 7.5). Two to 20 μg of extracted protein was assayed in a volume of 50 μl. The reaction was started by adding the ^{14}C-adenosine (final concentration 16 μM, 1.96 Gbq mmol^{-1}) or ^{3}H-isopentenyladenosine (85 nM, 666 Gbq mmol^{-1}, synthesized according to Laloue and Fox, (1987). Reactions were stopped by adding 250 μl of ethanol (2°C) containing non-radiolabelled adenosine and isopentenyladenosine (each 1 μg ml^{-1}).

When using ^{14}C-adenosine as a substrate, the nucleotides were precipitated with 2 ml of 80 mM lanthanium chloride during a 30-min incubation on ice (Chasin, 1974). Nucleotides were retained after filtering on glass fiber filters (Whatman no. 6). The filters were covered with 4 ml liquid scintillation cocktail (Pico Fluor 40, Packard, Dreieich, Germany) and radioactivity was measured. In order to confirm the identity of the reaction products, the assay mix was analysed by HPLC (anion exchange and reverse-phase chromatography) as described by Moffatt *et al.* (1991). For the assays with ^{3}H-isopentenyladenosine as substrate, the conversion to the corresponding nucleotides was quantified by HPLC.

In vivo feeding of Physcomitrella *with tritiated cytokinins*

Chloronema tissue of *Physcomitrella* was grown in liquid culture as described above. One-hundred milligrams of fresh weight were incubated in 4 ml of aerated liquid medium. Tritiated isopentenyladenosine (or isopentenyladenine) was added to a final concentration of 0.28 μM. After 45 and 90 min incubation the tissue was harvested and washed with fresh cold medium for a few seconds by filtration. To the tissue, 1 ml of Bieleski's reagent [methanol/chloroform/formic acid/water; 12/5/1/2 (v/v/v/v)] was added and cells were homogenized in a potter. The homogenate was stored at –20°C for 15 h. After centrifugation at 15 000 ***g***, 0.5 ml of distilled water was added to the supernatant in order to give a phase separation. The upper layer containing the cytokinins was analysed by HPLC liquid scintillation counting (Moffatt *et al.*, 1991) for tritiated cytokinin metabolites.

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References

Ashton, N.W. and Cove, D.J. (1977) The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants of the moss, *Physcomitrella patens. Mol. Gen. Genet.* **154**, 87–95.

Auer, C.A., Cohen, J.D., Laloue, M. and Cooke, T.J. (1992) Comparison of benzyladenine metabolism in two *Petunia hybrida* lines differing in shoot organogenisis. *Plant Physiol.* **57**, 15–22.

Barankiewicz, J. and Paszkowski, J. (1980) Purine metabolism in mesophyll protoplasts of tobacco (*Nicotiana tabacum*) leaves. *Biochem. J.* **186**, 343–350.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Burch, L.R. and Stuchbury, T. (1986) Metabolism of purine nucleotides in the tomato plant. *Phytochem.* **25**, 2445–2449.

Chasin, L.A. (1974) Mutations affecting adenine phosphoribosyl transferase activity in chinese hamster cells. *Cell*, **2**, 37–41.

Chen, C.-M. (1982) Biosynthesis and enzymic regulation of the interconversion of cytokinin. In *Metabolism and Molecular Activities of Cytokinins* (Guern, J. and Peaud-Leonel, C., eds). Berlin: Springer-Verlag, pp. 34–43.

Chen, C.-M. and Eckert, R.L. (1977) Phosphorylation of cytokinin by adenosine kinase from wheat germ. *Plant Physiol.* **59**, 443–447.

Chen, C.-M., Melitz, D.K. and Clough, F.W. (1982) Metabolism of cytokinin: phosphoribosylation of cytokinin bases by adenine phosphoribosyltransferase from wheat germ. *Arch. Biochem. Biophys.* **214**, 634–641.

Del Sal, G., Manfioletti, G. and Schneider, C. (1989) The CTAB-DNA precipitation method: a common mini-scale preparation of template DNA from phagemids, phages or plasmids suitable for sequencing. *BioTechniques*, **7**, 514–518.

Doree, M. and Terrine, C. (1973) Enzymatic synthesis of ribonucleoside-5′-phosphates from some N6-substituted adenosines. *Phytochem.* **12**, 1017–1023.

Dower, W.J., Miller, J.F. and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucl. Acids Res.* **16**, 6127–6145.

Guranowski, A. (1979) Plant adenosine kinase: purification and some properties of the enzyme from *Lupinus luteus* seeds. *Arch. Biochem. Biophys.* **196**, 220–226.

Guranowski, A. and Wasternak, C. (1982) Adenine and adenosine metabolizing enzymes in cell-free extracts from *Euglena gracilis. Comp. Biochem. Physiol.* **71**, 483–488.

Hove-Jensen, B. and Nygaard, P. (1989) Role of the guanosine kinase in the utilisation of guanosine for nucleotide synthesis in *Escherichia coli. J. Gen. Microbiol.* **135**, 1263–1273.

Laloue, M. and Fox, J.E. (1987) The synthesis of tritiated ribosylzeatin with high specific activity. *Phytochem.* **26**, 987–989.

Lee, D. and Moffatt, B. (1994) Adenine salvage activity during callus induction and plant growth. *Physiologia Plantarum*, **90**, 739–747.

Le Floc'h, F. and Faye, F. (1995) Metabolic fate of adenosine and purine metabolism enzymes in young plants of peach trees. *J. Plant Physiol.* **145**, 398–404.

McGaw, B.A. (1995) Cytokinin biosynthesis and metabolism. In *Plant Hormones* (Davis, P.J ed.). Dordrecht: Kluwer Academic Publishers, pp. 98–117.

Moffatt, B., McWhinney, E.A., Burkhardt, W.E., Pasternak, J.J. and Rothstein, S.J. (1992) A complete cDNA for adenine phosphoribosyltransferase from *Arabiopsis thaliana. Plant Mol. Biol.* **18**, 653–662.

Moffatt, B., Pethe, C. and Laloue, M. (1991) Metabolism of benzyladenine is impaired in a mutant of *Arabidopsis thaliana* lacking adenine phosphoribosyltransferase activity. *Plant Physiol.* **95**, 900–908.

Mok, D.W.S. and Martin, R.C. (1994) Cytokinin metabolic enzymes. In *Cytokinins – Chemistry, Activity and Function* (Mok, D.W.S. and Mok, M.C., eds). Boca Raton: CRC Press, pp. 129–137.

Nygaard, P. (1983) Utilisation of preformed purine bases and nucleosides. In *Metabolism of Nucleotides, Nucleosides and Nucleobases in Microorganisms* (Munch-Petersen, A. ed.). London: Academic Press, pp. 27–93.

Reski, R., Faust, M., Wang, X.-H., Wehe, M. and Abel, W.O. (1994) Genome analysis of the moss *Physcomitrella patens* (Hedw.) B.S.G. *Mol. Gen. Genet.* **244**, 352–359.

Reski, R., Reutter, K., Kasten, B., Faust, M., Kruse, S., Gorr, G., Strepp, R. and Abel, W.O. (1995) Molecular analysis of chloroplast division. In *Current Issues in Plant Molecular and Cellular Biology* (Terzi, M. *et al.,* eds). Dordrecht, Kluwer Academic Publishers, pp. 291–296.

Reski, R., Wehe, M., Hadeler, B., Marienfeld, J.R. and Abel, W.O. (1991) Cytokinin and light quality interact at the molecular level in the mutant PC22 of the moss *Physcomitrella. J. Plant Physiol.* **138**, 236–243.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning – A Laboratory Manual*. New York: Cold Spring Habour Press.

Sanger, F., Niklen, S. and Coulsen, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Schnorr, K.M., Gaillard, C., Biget, E., Nygaard, P. and Laloue, M. (1996) A second form of adenine phosphoribosyltransferase in *Arabidopsis thaliana* with relative specificity towards cytokinins. *Plant J.* **9**, 891–898.

Short, J.M., Fernandez, J.M., Sorge, J.A. and Huse, W.D. (1988) λ ZAP: a bacteriophage λ expression vector with *in vivo* excision properties. *Nucl. Acids Res.* **16**, 7583–7600.

Spychala, J., Datta, N.S., Takabayashi, K., Datta, M., Fox, I.H., Gribbin, T. and Mitchell, B.S. (1996) Cloning of human adenosine kinase cDNA: sequence similarity to microbial ribokinases and fructokinases. *Proc. Natl Acad. Sci. USA*, **93**, 1232–1237.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Custal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22**, 4673–4680.

Wang, T., Cove, D.J., Beutelmann, P. and Hartmann, E. (1980) Isopentenyladenine from mutants of the moss, *Physcomitrella patens. Phytochem.* **19**, 1103–1105.

Wang, T., Horgan, R. and Cove, D. (1981) Cytokinins from the moss *Physcomitrella patens*. *Plant Physiol*. **68**, 735–738.

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