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Two thymidine kinases and one multisubstrate deoxyribonucleoside kinase salvage DNA precursors in *Arabidopsis thaliana*

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Deoxyribonucleotides are the building blocks of DNA and can be synthesized via *de novo* and salvage pathways. Deoxyribonucleoside kinases (EC 2.7.1.145) salvage deoxyribonucleosides by transfer of a phosphate group to the 5′ of a deoxyribonucleoside. This salvage pathway is well characterized in mammals, but in contrast, little is known about how plants salvage deoxyribonucleosides. We show that during salvage, deoxyribonucleosides can be phosphorylated by extracts of *Arabidopsis thaliana* into corresponding monophosphate compounds with an unexpected preference for purines over pyrimidines. Deoxyribonucleoside kinase activities were present in all tissues during all growth stages. In the *A. thaliana* genome, we identified two types of genes that could encode enzymes which are involved in the salvage of deoxyribonucleosides. Thymidine kinase activity was encoded by two thymidine kinase 1 (EC 2.7.1.21)-like genes (*AtTK1a and AtTK1b*). Deoxyadenosine, deoxyguanosine and deoxycytidine kinase activities were encoded by a single *AtdNK* gene. T-DNA insertion lines of *AtTK1a* and *AtTK1b* mutant genes had normal growth, although *AtTK1a AtTK1b* double mutants died at an early stage, which indicates that AtTK1a and AtTK1b catalyze redundant reactions. The results obtained in the present study suggest a crucial role for the salvage of thymidine during early plant development.

Database
Sequence data from the present study have been deposited in the EMBL database/GenBank under accession numbers: AT3G07800.1 (*AtTK1a*), At5G23070.1 (*AtTK1b*) and AT1G72040.1 (AtdNK).

Introduction

Living organisms need a balanced supply of DNA precursors to replicate their DNA and the enzymatic reactions that are involved are vital for any cell. Deoxyribonucleosides (dNs) and deoxyribonucleotides (dNts) can be synthesized via the *de novo* or salvage pathways. Ribonucleotide reduction by ribonucleotide reductase (RNR) is the rate limiting step in the *de novo* pathway and the salvage pathway is controlled

Abbreviations
BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; dAK, deoxyadenosine kinase; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; dN, deoxyribonucleoside; dNK, deoxyribonucleoside kinase; dNts, deoxyribonucleotides; MS, Murashige and Skoog; RNR, ribonucleotide reductase; TK, thymidine kinase.

by deoxyribonucleoside kinases (dNKs) (EC 2.7.1.145), which convert dNs into corresponding 5′-monophosphate dNs [1–3]. The dNts can subsequently be converted into corresponding di- and tri-phosphates by nucleotide kinases and incorporated into the newly-synthesized DNA strand by DNA polymerases.

dNKs are well characterized in unicellular eukaryotes [4], insects [5] and mammals [2]. However, these enzymes are not present in yeast and fungi, and the genes coding for enzymes in the salvage pathway have presumably been lost in this lineage during evolution [3]. In the unicellular amoeba *Dictyostelium discoideum*, there are three dNKs: thymidine kinase 1 (TK1) (EC 2.7.1.21), deoxyadenosine kinase and deoxyguanosine kinase [4]. Insects have only a single multisubstrate enzyme expressing all four dNK activities [5]. Humans have four different dNKs: TK1 and deoxycytidine kinase (dCK), which are mainly located in the cytosol, and thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK), which are found in the mitochondria. The mitochondrial dNKs are essential for the replication and repair of mitochondrial DNA because no RNR activity is present in the human mitochondria. However, although mice deficient in TK1 [6], TK2 [7] or dCK [8] are severely sick, they can pass the developmental stage and reach the adult stage.

The nucleotide precursor metabolism is poorly understood in plants. Salvage of pyrimidines has been described in *Pisum sativum* [9], *Solanum tuberosum* [10], *Pinus radiata* [11] and *Picea glauca* [12]. In *A. thaliana*, it was demonstrated that the response to mutagens and proper plant development was dependent of RNR [13] and that RNR and TK1-like genes were transcribed during genotoxic stress [14]. Salvage of uracil [15] and uridine [16] was shown to be important in early plant development and, furthermore, the degradation of uridine might be important for germination and plant development [17]; however, it was also demonstrated that the same two nucleoside hydrolases are unimportant for seedling germination and development [18]. Recently, a nucleobase importer was shown to provide substrates for the essential salvage of pyrimidines [19]. A unique TK1 with the capability to phosphorylate dN monophosphate was isolated from tomato [20], and in a preliminary study, one dNK in *A. thaliana* was also reported [21].

In the present study, we aimed to characterize the molecular basis for salvage of dNs in *A. thaliana* through biochemical and genetic analysis. We show that *A. thaliana* can salvage dNs and also that two types of dNK genes are present in *A. thaliana*: two TK1-like enzymes and a single TK2-like enzyme. The two TK1-like genes codes for enzymes with thymidine kinase activity, whereas the TK2-like gene codes for a multisubstrate dNK with broad specificity towards deoxyadenosine, deoxyguanosine and deoxycytidine (but not thymidine). We also report that mutants in both TK1-like genes are phenotypically normal, although a TK1 double mutant died at an early stage.

Results

A. thaliana phosphorylates all native deoxyribonucleosides

Initially, we measured the dNK activities in *A. thaliana* buds and siliques using radiolabelled dNs as substrates and ATP as the phosphate donor. The highest activity was found in the buds with dA and dG. However, dC and dT were also phosphorylated but to a lower extent (Fig. 1). A similar relative distribution between the four activities was observed in green immature siliques, although the activities were slightly lower. The activities in seeds were also monitored during culture in liquid medium and after 24 h, the observed dNK activities were low (Fig. 2A). Over subsequent days, the dNK activities increased to almost constant levels. A peak of the dCK and TK activities could be seen at day 4. Deoxyadenosine kinase (dAK) activity increased daily with a peak at day 7, whereas dGK activity was relatively constant during the time course of the study. We also monitored dNK activities during the growth of a cell culture for 8 days after inoculation, although the activities did not change much, even if the growth rate of the cells peaked at day 4 (Fig. 2B).

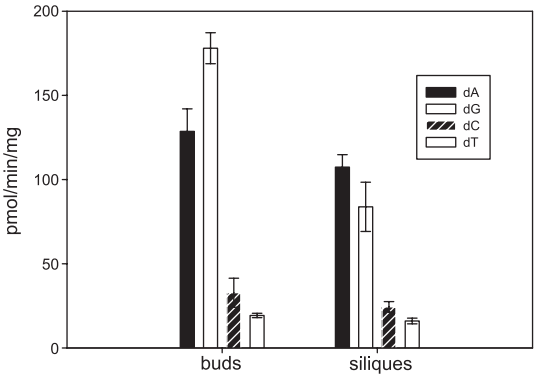

Fig. 1. Deoxyribonucleoside kinase activities in two plant organs. Protein extracts of green immature siliques and buds from 1-month-old plants of *A. thaliana* were analyzed for their ability to phosphorylate the four natural deoxyribonucleosides (dT, dA, dG and dC) at 200 μM. Activities with purines dNs are higher than with pyrimidine dN substrates.

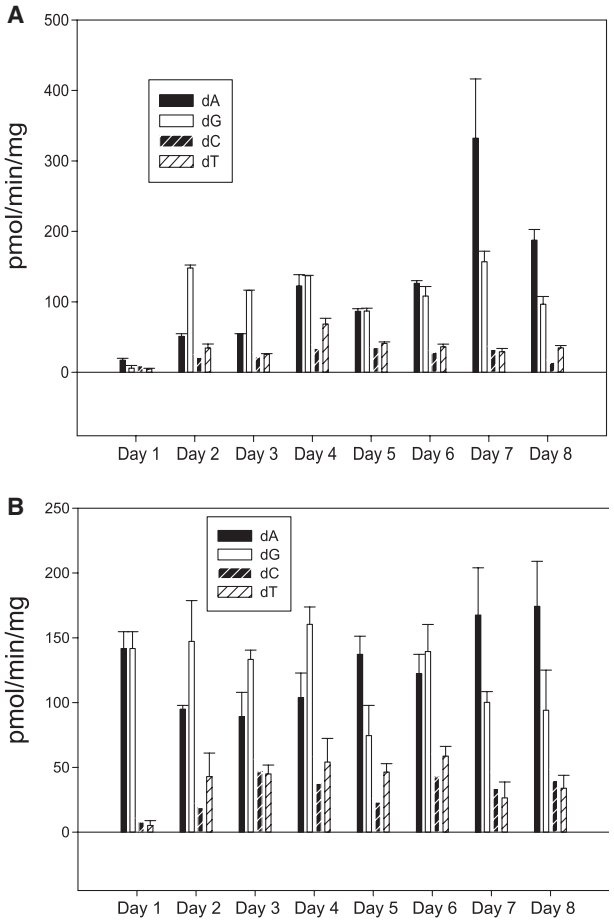

Fig. 2. Time course study on TK, dCK, dAK and dGK activities during the first days of plant growth. (A) The four dN kinase activities on seedlings after subculture at day 0. (B) The four dN kinase activities on a *A. thaliana* cell culture of the ecotype Landsberg after subculture at day 0. dN kinase activities were determined with 200 μM of the natural substrates dT, dC, dA and dG. The results are obtained from three independent experiments. Error bars represent the SD.

Two TK1-like kinases are present in the genome

Based on homology with human TK1, two different GeneBank entries, accession numbers AAF13097 and BAB09824, for *A. thaliana* were identified. AAF13097 (gene locus AT3G07800) predicted an ORF of 717 bp, resulting in a protein of 238 amino acids with a calculated molecular mass of 26.1 kDa (AtTK1a). BAB09824 (gene locus AT5G23070) predicted an ORF of 834 bp, resulting in a protein of 277 amino acids with a calculated molecular mass of 30.7 kDa (AtTK1b). The amino acid identity between the AtTK1a and AtTK1b proteins was 62%. The N-terminal, however, was extended in the AtTK1b protein and contained a putative organelle localization signal (Fig. S1). An N-terminal deleted mutant with the first 45 amino acids was constructed and named AtTK1b-dN45. It should be noted that the genomic sequence of AtTK1b, in contrast to AtTK1a, does not contain any introns. Multiple alignments of the plant AtTK1a, AtTK1b-dN45 and AtdNK amino acid sequences, together with other described dNKs, are shown in Figs S1 and S2. A phylogenetic analysis of a few characteristic TK1 and the two *A. thaliana* TK1s described in the present study showed that the two plant TK1s were not duplicated very recently, although this definitely occurred after the split of animal and plant TK1s (Fig. 3).

TK1 activity is essential

In the SALK T-DNA insertion collection library (Table S1), we identified two putative mutant lines of *AtTK1a* and one putative mutant line of *AtTK1b* that had the T-DNA insertion in exons. We confirmed the insertion of the T-DNA by gene specific primers (Table S1). We could not observe any effect on plant growth for these three mutants (Fig. 4A–C). In addition, the measured TK1 and dAK activities were similar to that observed in wild-type plants (data not shown).

We crossed two homozygous mutants of *AtTK1a* (*AtTK1a-1::T-DNA* and *AtTK1a-2::T-DNA*) with a homozygous mutants of *AtTK1b* (*AtTK1b::T-DNA*) to generate double mutants, (*AtTK1ab-1::T-DNA*) and (*AtTK1ab-2::T-DNA*), lacking both functional TK1 genes. The F_1 generation was heterozygous at both loci, $AtTK1a^{+}/AtTK1a^{-}$ and $AtTK1b^{+}/AtTK1b^{-}$, and these lineages behaved similar to the wild-type. We screened over 100 independent plants from the F_2 populations on Murashige and Skoog (MS) plates for their genotype (Table S2). We identified 12 out of 116 plants of the *AtTK1ab-1::T-DNA* mutant line and seven out of 110 plants of the *AtTK1ab-2::T-DNA* mutant parental line (Fig. 4) that were unable to develop into a mature plant. These evenly whitish seedlings did not develop true leaves and only survived a short period *in vitro*. PCR analysis of these whitish small plants (maximum size of 3 mm) showed that they had homozygous mutations in both the *AtTK1a* and *AtTK1b* genes. Therefore, we characterized this double mutation as lethal. In addition, we observed 13 plants out of 89 in the *AtTK1ab-1::T-DNA* cross and 11 out of 81 in the *AtTK1ab-2::T-DNA* cross that had variegated leaves (Fig. S3). This variegation was only visible up to 3 weeks and, subsequently, these leaves turned green and the plants had a delayed flowering. These plants produced normal amounts of seeds. PCR analysis of genomic DNA from these plants revealed

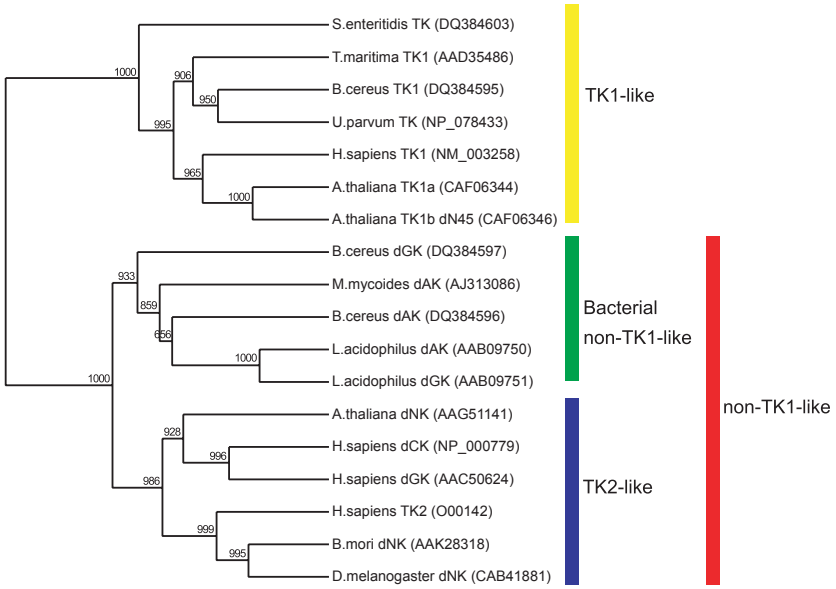

Fig. 3. Phylogenetic relationship among deoxyribonucleoside kinases. Phylogenetic clustering tree of the dNKs described in the present study together with other known dNKs from eukaryotes and prokaryotes. dNKs can be separated in two large groups: the TK1-group and the non-TK1-group. The TK1-group can further be subgrouped into a human-TK1-group and a non-human-TK1-group comprising TK1 enzymes from Gram-negative bacteria. The non-TK1-group can also be subgrouped into a eukaryotic non-TK1-like-group and a bacterial non-TK1-group. The putative signal sequence containing 45 amino acids were deleted in the N-terminus of AtTK1b. The numbers at the nodes are the percentage of frequencies with which a given branch appeared in 1000 bootstraps replicates. Note that AtdNK clusters with the animal dCK and dGK. Further phylogenetic analysis is provided elsewhere [27].

that they were homozygous at the *AtTK1b-1::T-DNA* locus but heterozygous at *AtTK1a-1::T-DNA*.

Both TK1s have the same substrate specificity

We subcloned, over-expressed and purified the plant ORFs as glutathione *S*–transferase-fusions encoding putative *A. thaliana* AtTK1a, AtTK1b, AtTK1b-dN45 (which was missing the 45 N-terminal residues) and AtdNK. Recombinant enzymes were purified by affinity-chromatography. The kinetic parameters of these purified recombinant enzymes were studied (Table 1). AtTK1a, AtTK1b and the AtTK1b-dN45 phosphorylated dU, dT and the antiviral drug 3′-azido-3′-deoxythymidine (Fig. S4) and hyperbolic Michaelis–Menten kinetics were observed with all of the dNs tested.

Only one non-TK1-like is present

When deposited sequences from *A. thaliana* were analyzed for homology to DmdNK, a putative dNK with a complete ORF of 1086 bp, resulting in a protein of 361 amino acids with a calculated mass of 41.2 kDa, was found (accession number: AAg51141, gene locus AT1G72040). We were unable to identify any other putative dNKs in the genome. The phylogenetic relationship between the plant dNKs and other dNKs from eukaryotes, as well as from prokaryotes, is presented in Fig. 3, and shows that AtdNK groups together with human dCK and dGK (Fig. 3) but branches out before the split of the two animal kinases.

AtdNK is a multisubstrate dNK

Initial attempts to purify recombinant AtdNK were unsuccessful because all of the dNK activity was lost during the purification. Therefore, several buffers and combinations of varying concentrations of Triton X-100, glycerol, $MnCl_2$, $MgCl_2$, EDTA, $CaCl_2$, benzamidine, aminocaproic acid, GTP, ATP, TTP or NaPPP (sodium pyrophosphate) were investigated for their effect on the activity and stability (Fig. S5). Clearly, Tris-buffer was poorer than the phosphate buffer. The addition of 2 mM ATP and 2 mM $MgCl_2$ to the standard NaCl/P_i buffer increased the activity in the lyzed extract by approximately fivefold, although it had no stabilizing effect during storage for 24 h at −80 °C. However, the addition of 2 mM $MnCl_2$ to the NaCl/P_i buffer containing 2 mM ATP and 2 mM $MgCl_2$ not only increased the activity in the lyzed extract by two-fold, but also had a remarkably stabilizing effect, which was maintained 100% during

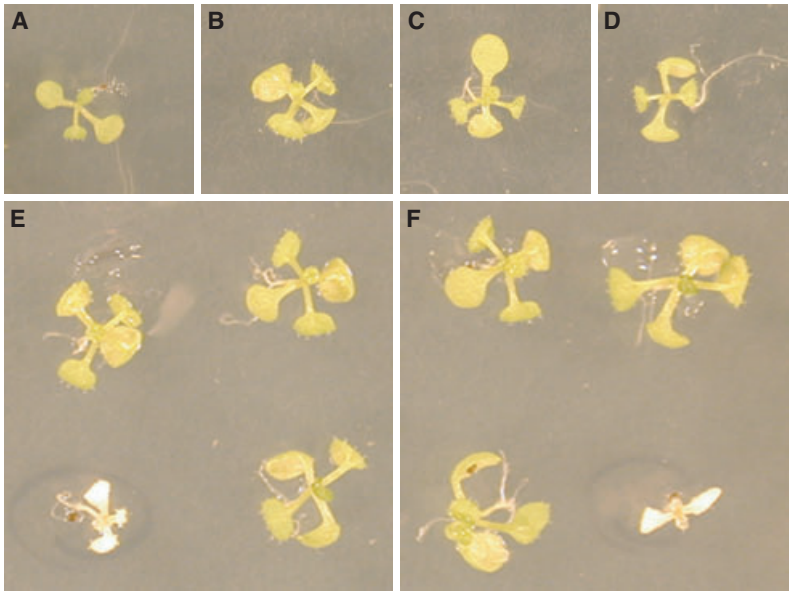

Fig. 4. Seedlings of homozygous mutant lines with mutated kinase genes. Top left to right: (A) *AtTK1a-1::T-DNA*, (B) *AtTK1a-2::T-DNA* and (C) *AtTK1b-1::T-DNA* homozygous mutant lines, respectively, and (D) wild-type. Flowering time, number of leaves, size of leaves and seed production were monitored, although there was no difference in appearance and growth between these plants and wild-type. In addition, the TK activities were close to that of the wild-type. Examples from the F_2 populations originating from crosses for the generation of double TK1 mutant lines: (E) *AtTK1ab-1::T-DNA* to the bottom left and (F) *AtTK1ab-2::T-DNA* to the bottom right. The whitish plants are double mutants, according to PCR analysis, and were unable to develop into adult plants.

storage for at least 6 months at –80 °C. Adding 2 mM ATP, 2 mM $MgCl_2$ and 2 mM $MnCl_2$ to the chromatography buffer made it possible to purify the enzyme with a good recovery.

AtdNK had a broad substrate specificity and could phosphorylate dA, dG and dC with K_m values in the range 14–26 μM, whereas the K_m for dU was 561 μM. Positive cooperativity was found with several substrates (Table 2 and Fig. S4). Despite the efficient

Table 1. Kinetic parameters of the recombinant plant TK1 enzymes. The data for HsTK1 are also shown [35]. AZT, 3′-azido-3′-deoxythymidine.

	K_M (μM) (±SD)	k_{cat} (s^{-1}) (±SD)	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
AtTK1a			
dU	15 ± 2	1.5 ± 0.1	1.5×10^5
dT	0.62 ± 0.08	0.48 ± 0.03	7.7×10^5
AZT	2.7 ± 0.8	0.13 ± 0.03	4.8×10^4
AtTK1b			
dU	6.6 ± 0.5	0.045 ± 0.006	6.8×10^3
dT	1.4 ± 0.4	0.020 ± 0.002	1.4×10^4
AZT	0.38 ± 0.04	0.009 ± 0.002	2.2×10^4
AtTK1bdN45			
dU	5.6 ± 1.3	0.30 ± 0.07	5.3×10^4
dT	0.84 ± 0.19	0.20 ± 0.09	2.4×10^5
AZT	1.9 ± 0.4	0.22 ± 0.03	1.2×10^5
HsTK1			
dU	9.0	5.0	5.6×10^5
dT	0.5	4.0	8.0×10^6
AZT	0.6	2.1	3.5×10^6

Table 2. Kinetic parameters of the recombinant plant AtdNK. The data for HsTK2 [35], HsdCK [37] and HsdGK [38] are also shown. NA, no activity; *h*, Hill constant. *The two $K_{0.5}$ values were obtained at substrate concentrations below or above 8–10 μM substrate.

	$K_{0.5}$ (μM) (± SD)	k_{cat} (s^{-1})	$k_{cat}/K_{0.5}$ ($s^{-1}\cdot M^{-1}$)	*h*
AtdNK				
dA	14.2 ± 0.7	0.47 ± 0.17	3.3×10^4	1.0
dC	16.3 ± 1.0	0.29 ± 0.07	1.8×10^4	1.9 ± 0.07
dG	26.3 ± 1.8	0.27 ± 0.08	1.0×10^4	2.0 ± 0.07
dFdC	159 ± 20	0.77 ± 0.02	4.8×10^3	1.0
BVDU	113 ± 16	0.105 ± 0.003	9.3×10^2	1.0
dT	0	0	0	NA
dU	561 ± 105	0.23 ± 0.06	4.1×10^2	1.0
HsdCK				
dA	120	0.32	2.7×10^3	1
dC	1	0.074	7.4×10^4	1
dG	150	0.41	2.7×10^3	1
HsTK2				
dT	0.3; 16*	0.29	9.6×10^5	1
dC	36	0.47	1.3×10^4	1
HsdGK				
dA	60	1.5	2.5×10^4	1
dG	36	2.3	6.4×10^4	1

phosphorylation of dU and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), dT could not be phosphorylated.

Discussion

The salvage of dNs in plants has been poorly described. In the present study, we report that *A. thaliana* can salvage dNs and we isolated and characterized the responsible enzymes. Extracts of plants from early growth to mature plant were analyzed for phosphorylating capacity towards all four natural dNs and it is shown that all four dNs can be phosphorylated with a preference towards purines over pyrimidines. The dNK activities were at the same level in developing organs such as buds and green immature siliques (Fig. 1), plant seedlings (Fig. 2A) and plant cell cultures (Fig. 2B), indicating that dNK activities were not limited to a specific cell tissue or developmental stage. The low thymidine kinase activity in the developing cells and tissues was unexpected compared to the mammalian cells, which have high TK1 activity in dividing cells [22–24]. The dNK in the fruit fly is also cell cycle regulated, although the demand for dNTPs and the regulation of the nucleotide pool in plants is apparently different from that in mammals [25].

We describe that two TK1-like enzymes (AtTK1a and AtTK1b) and a single TK2-like enzyme (AtdNK) are encoded by the *A. thaliana* genome. Two extra *tk1* genes have also been found in mice, although both psedogenes are inactive *in situ* [26]. The phylogenetic relationship with human TK1 suggests that the duplication took place after the separation of the lineages leading to plants and mammals (Fig. 3). AtTK1b is longer in its N-terminal sequence than AtTK1a and its human homologue. Longer N-terminals fragments are also found in human TK2 and dGK, which are located in the mitochondria, suggesting that AtTK1b could be located in plastids and maybe mitochondria. Preliminary analysis suggests that *A. thaliana* mitochondria are responsible for a major part of the observed dNK activity [27].

Single gene mutants of TK1, dCK or TK2 result in severe phenotypes in mice; however, single gene mutants of *AtTK1a* and *AtTK1b* could grow normally, indicating that the genes do not encode essential activities (Fig. 4A–C). The lack of any visible phenotype in the single gene mutants, either *AtTK1a* or *AtTK1b*, could have two explanations: (a) thymidine salvage has a minor role in *A. thaliana* or (b) the genes for TK1a and TK1b are redundant. To test the second hypothesis, *AtTK1a AtTK1b* double mutants were generated and analyzed. The appearance of variegated plants from the F_2 generation of plants that were homozygous at the *AtTK1b* locus (Fig. S3E) but heterozygous at the *AtTK1a* locus indicates that the loss of the *AtTK1b* gene gives a stronger phenotype than the loss of the *AtTK1a* gene because we did not detect any growth phenotypes on plants homozygous for the *AtTK1a* mutation and heterozygous for the *AtTK1b* mutation. By contrast, double mutants having mutations in both *AtTK1* genes could not develop into fully growing plants, suggesting that at least one copy of the *TK1* gene is necessary to complete plant development.

TK1 from humans can only phosphorylate dT and dU but no other natural dN, and similarly recombinant purified AtTK1a and AtTK1b also have narrow substrate specificity. A 45 amino acid deletion of the N-terminal of AtTK1b results in an enzyme with the same K_m as the full-length product but an approximately ten-fold higher k_{cat}, supporting the idea that those 45 amino acids represent a signal peptide that needs to be processed before AtTK1b is fully functional.

AtdNK is phylogenetically related to the TK2 family and could represent a model for deducing a common ancestor to human dCK and dGK enzymes. AtdNK is larger than the human mammalian counterparts and has one extra segment of 32 amino acids close to the C-terminal and an extended C-terminal of ~ 55 amino acids (Fig. S2). The biological role of these extra segments remains unknown. Purification of AtdNK has been complicated as a result of low stability during purification. Thus, to be able to purify this dNK, we set a screen aiming to identify a suitable buffer. We found that the addition of 2 mM ATP, 2 mM $MnCl_2$, 2 mM $MgCl_2$ and 1% Triton X-100 increased the activity by almost twenty-fold, and also that the use of this buffer facilitated the purification of recombinant AtdNK (Fig. S5), with $MnCl_2$ apparently being essential for the *in vitro* stability for this dNK. AtdNK has a broad substrate specificity and was able to phosphorylate deoxyadenosine, deoxyguanosine and deoxycytidine (but not thymidine), supporting the idea that AtdNK could represent a common ancestor to human dGK and dCK, a nonspecialized dNK with a broad specificity. In addition AtdNK could efficiently phosphorylate nucleoside analogues such as difluorodeoxycytidine and BVDU. The phosphorylation of dU and BVDU (but not dT) suggests that there is a specific steric hinderance for a methyl group on the base, although apparently not for a bromovinyl group. A high resolution structure of this enzyme would be necessary to explain the findings reported in the present study.

Materials and methods

Biological material

Arabidopsis thaliana ecotype Columbia-0 was used as the wild-type plant and a green cell culture from *A. thaliana* ecotype Landsberg erecta was used for the *in vivo* measurements of dNK activities [28]. *Escherichia coli* XL-1 (Stratagene, La Jolla, CA, USA) was used for cloning and the TK negative *E. coli* KY895 (F^-, *tdk*$^-$, *ilv*) was used for expression of the recombinant dNKs [29]. T-DNA insertion lines were obtained from the SALK collection [30] and confirmed as homozygous mutants using gene- and T-DNA-specific primers (Table S1). We obtained two kind of insertions in the *TK1a* gene (referred to as gene A). Therefore, we had a1 and a2 deletion alleles, designated *AtTK1a-1::T-DNA* and *AtTK1a-2::T-DNA*, respectively. Another insertion line was obtained, designated *AtTK1b-1::T-DNA*, in the TK1b gene (referred to as gene B). The second T-DNA mutant with insert in *AtTK1b* (GK-14D03) was also investigated, although we were only able to recover wild-type from that seed batch (Table S1). We isolated homozygous mutants for the *TK1a* gene (aa) and *TK1b* gene (bb). The 'aa' and 'bb' lineages were crossed and generated the F_1 generation (AaBb), which was then self-crossed to generate the F_2 populations (Table S2).

Chemicals

Unless stated otherwise, chemicals were of the highest purity and were obtained either from Sigma (St Louis, MO, USA) or Merck (Damstadt, Germany). All ^{3}H-labelled nucleosides were from Moravek Biochemicals Inc. (Brea, CA, USA), except ^{3}H-labelled dT, which was obtained from GE Healthcare (Little Chalfont, UK). dFdC was obtained from Thykn (Mumbai, India). Thrombin was obtained from Biofac (Kastrup, Denmark).

Cultivation of plant cell material

Plant cell culture was subcultured in the medium containing 1 × Murashige and Skoog (MS) basal salt mix [31], 1 × Gamborg's B_5 vitamins and 3% sucrose, with pH adjusted to 5.7, and, after autoclaving, 0.5 mg·L^{-1} naphthalene acetic acid and 0.05 mg·L^{-1} N^6 benzyl adenine was added. In total, 3 mL of the 1-week culture was transferred in 100 mL of fresh medium and cultivated at 22 °C under a 16 : 8 h light:dark cycle. *A. thaliana* seeds (ecotype Columbia) for liquid seedling culture were inoculated into 100 mL of medium (0.5 × MS medium, 2% sucrose, pH 5.8) and cultivated at 22 °C under a 16 : 8 h light:dark cycle. For plant growth experiments, surface sterilized *A. thaliana* seeds were sown on MS plates.

Protein extraction from plant cells and tissues

Arabidopsis thaliana (ecotype Columbia) for preparations of siliques and buds were grown in soil under standard greenhouse conditions at 23 °C with ~ 16 h light per day. Green siliques and buds were harvested on 4-week-old plants. In total, 1–5 g of plant material and ice-cold plant extraction buffer (1 x NaCl/P_i, 5 mM dithiothreitol, 0.1% Triton X-100, 6 mM NaF, 2 mM ATP, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 10% glycerol, 0.2 mM phenylmethanesulfonyl fluoride, 0.01 mM leupeptin, 1 nM calyculin and 0.5% PVP 40) were ground with sand in mortars pretreated with liquid nitrogen. The cell culture was filtered through a 30-μm nylon net and plant extraction buffer was added. Subsequently, the culture was thawn and frozen three times in an ultrasound bath and liquid nitrogen, respectively. Plant tissues and cell culture were incubated for 10 min at 4 °C with shaking and then centrifuged for 5 min at 18 000 ***g*** in a JA-20 rotor (Beckman Coulter, Fullerton, CA, USA) at 4 °C; supernatants were frozen at −80 °C.

Cloning of genes encoding plant kinases

The ORFs were amplified from a cDNA library obtained from Stratagene by PCR using specific primers with overhang for cloning and then cloned into pGEX-2Ts expression vector (Amersham Pharmacia Biotech/GE Healthcare, Uppsala, Sweden). In addition, primers were designed to construct N-terminal deletions of 22, 45 and 63 amino acids. The constructs harbouring AtTK1a, AtTK1b, AtTK1b-dN22, AtTK1b-dN45, AtTK1b-dN63 and AtdNK were named P604, P661, P662, P663, P664 and P605, respectively, and sequenced using a commercial source (MWG-Biotech, Ebersberg, Germany).

Phylogenetic analysis

Amino acid sequence alignments were performed using CLUSTALW [32]. The phylogenetic analysis was performed using TREECON, version 1.3b [33] by means of clustering and neighbourhood-joining calculation approaches.

Expression and purification of plant dNKs

Recombinant AtTK1a, AtTK1b and AtTK1b-dN45 were expressed and purified as described previously [34]. AtdNK was also purified in accordance with this procedure but with 2 mM $MgCl_2$, 2 mM $MnCl_2$ and 2 mM ATP added to all buffers to stabilize the enzyme.

dNK enzyme assays

dNK activities were determined by initial velocity measurements based on four time samples by the DE-81 filter paper

(Whatman International, Maidstone, UK) assay using various radiolabelled/labelled dN substrate concentrations [35]. For determination of dNK activities in *E. coli* cell extracts, 3 mM NaF was added to the reaction mixture and, when dC was used as substrate, 0.5 mM of the cytidine deaminase inhibitor tetrahydrouridine was added. One unit (u) of dNK activity is defined as the formation of the corresponding monophosphate product at at a rate of 1 nmol·min^{-1}. Kinetic data were evalulated by nonlinear regression analysis using the Michaelis–Menten equation $v = V_{max} \times [S]/(K_m + [S])$ or the Hill equation $v = V_{max} \times [S]^h/(K_{0.5}^h + [S]^h)$ as described previously [36].

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Supporting information

The following supplementary material is available:
Fig. S1. Multiple amino acid sequence alignments of the AtTK1a and AtTK1b described in the present study together with some other known TK1s originating from eukaryotes and prokaryotes.
Fig. S2. Multiple amino acid sequence alignment of characterized eukaryotic non-TK1 kinases, including AtdNK.
Fig. S3. Fourteen-day-old plants.
Fig. S4. Kinetic parameters of the recombinant plant kinases with different substrates.
Fig. S5. Stability of AtdNK in different buffers.
Table S1. SALK lines with mutated *TK1a* and *TK1b* used in the present study.
Table S2. Genotyping of F_2 populations from Parental crosses between homozygous single mutants at the TK1 loci (the TK1a and TK1b gene).

This supplementary material can be found in the online version of this article.

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