METABOLISM OF PURINE NUCLEOSIDES AND BASES IN SUSPENSION-CULTURED *ARABIDOPSIS THALIANA* CELLS

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Keywords: Purine nucleotides, Biosynthesis, Salvage, Catabolism, *Arabidopsis thaliana*.

We study the metabolism of purine nucleosides and purine bases in suspension-cultured cells of the model plant *Arabidopsis thaliana*. [8-^{14}C]Adenosine, [8-^{14}C]guanosine, [8-^{14}C]inosine, [8-^{14}C]xanthosine, [8-^{14}C]adenine, [8-^{14}C]guanine, [8-^{14}C]hypoxanthine and [8-^{14}C]xanthine were administered to cells in the cell division phase, and the uptake and metabolic fate of these compounds were monitored for 4 h. The rates of uptake of most purines were within the range 60-70 nmol gFW^{-1}. Xanthine and xanthosine were taken up more slowly. The rate of uptake was ordered as hypoxanthine > adenine > inosine > guanosine > guanine > adenosine > xanthosine > xanthine. A large amount of radioactivity from [8-^{14}C]adenosine, [8-^{14}C]guanosine, [8-^{14}C]adenine and [8-^{14}C]guanine, and a limited amount from [8-^{14}C]inosine and [8-^{14}C]hypoxanthine, was incorporated into nucleotides and RNA. The so-called purine salvage pathways of adenosine, guanosine, adenine, guanine, inosine and hypoxanthine are therefore functional in *A. thaliana*. These tracer experiments also reveal that significant amounts of these compounds were converted to xanthine, and enter the catabolic pathway via allantoin. Neither xanthosine nor xanthine is used in the synthesis of nucleotides and RNA. These compounds are entirely catabolized via allantoin and allantoic acid. Deamination of adenine and guanine rings takes place at the stage of AMP deaminase and guanosine deaminase, respectively. The pattern of purine metabolism in *A. thaliana* is similar to that in other plants. Adenine salvage activity estimated from the metabolism of [8-^{14}C]adenine, the cellular concentration of ATP, and expression of the *APT1* gene encoding adenine phosphoribosyltransferase all increased markedly at the lag phase of cell proliferation. These observations imply that the salvage pathway is important during the early stages of cell culture in *A. thaliana*.

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Introduction

The purine nucleosides adenosine, guanosine, inosine and xanthosine, and the purine bases adenine, guanine, hypoxanthine and xanthine, are produced by the degradation of purine nucleotides, AMP and GMP. Fig. 1 shows the structures of the purine nucleosides and bases used in this study. Some of these purine compounds are utilized for re-generation of nucleotide synthesis by the salvage pathways; the rest are catabolised by the purine catabolic pathways.[1-3] *Arabidopsis thaliana* is a model plant, for which the genome has been fully sequenced,[4] and it is useful in studying the metabolic function of purine compounds at the molecular level.

Only a few studies on purine nucleoside and purine base metabolism in *A. thaliana* have been carried out to date.[2,3,5-7] Many systematic studies of genomics, proteomics and metabolomics have been undertaken recently in *A. thaliana*,[8] and the present dynamic profiling of *in situ* metabolism of exogenously supplied ^{14}C-purine compounds will shed further light on the overall metabolism of purine compounds *in planta*.

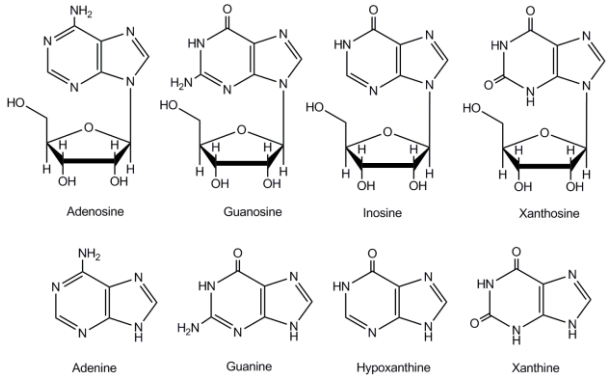

Figure 1. Structures of purine nucleosides and bases.

We have reported the *in situ* metabolism of ^{14}C-purine nucleosides and ^{14}C-purine bases in various plant materials, including cultured cells of *Catharanthus roseus*,[9] *Picea glauca*,[10] *Sonneratia alba*,[11] and *Bruguiera sexangula*,[12] and in tissue segments of *Phaseolus mungo* (=*Phaseolus aureus*),[13] *Solanum tuberosum*,[14] *Coffea arabica*,[15] *Theobroma cacao*[16] and *Camellia sinensis*.[17] Riegler *et al*.[6] recently reported their findings of *in situ* metabolism of [8-^{14}C]inosine and [8-^{14}C]xanthosine in roots of intact *A. thaliana* seedlings, as part of their study on nucleosidases. They argued that *A. thaliana* plants are able to salvage xanthosine for nucleic acid synthesis.[6] This novel finding differs from our previous observations in various plant species. As a result, it is important to determine whether purine metabolism is different in *A. thaliana* and in other plant species, especially because *A. thaliana* is a common model plant.

In the present study, the metabolism of labelled purine compounds was investigated using the culture system of *A. thaliana* which is suitable for our metabolic studies.[18,19] The metabolic fate of four purine ribonucleosides, [8-^{14}C]adenosine, [8-^{14}C]guanosine, [8-^{14}C]inosine and [8-^{14}C]xanthosine and four purine bases, [8-^{14}C]adenine, [8-^{14}C]guanine, [8-^{14}C]hypoxanthine and [8-^{14}C]xanthine, was investigated in the exponential growth phase of cells of *A. thaliana*.

Fluctuations in the *in situ* metabolism of [8-^{14}C]adenine and [8-^{14}C]inosine and the intracellular purine nucleotide level during culture growth were also examined. Finally, the expression of genes that encode enzymes involved in adenine and adenosine salvage was investigated. Our results indicate that the metabolism of purine nucleosides and bases in *A. thaliana* is essentially the same as in other non-purine alkaloid forming plant spices, such as *C. roseus* and *S. tuberosum*.

Materials and Methods

Plant material

Suspension cell-cultures of *A. thaliana*, accession Columbia (Strain T87), were obtained from the Experimental Plant Division of the RIKEN Bioresource Center, Tsukuba, Japan. The cell cultures used in this study are the same as in our previous study on phosphoribosylpyrophosphate synthetase.[18] In the growth experiments, the cells were sub-cultured at 10-day intervals in 25 ml of JPL medium, as in the work of Axelos et al.,[20] which contained 1 mM naphthaleneacetic acid and 1.5 % sucrose, in 100-ml Erlenmeyer flasks. The culture flasks were held on a horizontal rotary shaker (120 strokes min^{-1}, amplitude 80 mm) in a dedicated plant growth room at 22 ºC with a 16 h : 8 h light : dark cycle. Chlorophyll content was determined according to the method of Bruinsma.[21]

Radiochemicals and biochemicals

We obtained [8-^{14}C]adenosine (specific activity 2.0 GBq mmol^{-1}), [8-^{14}C]guanosine (specific activity 1.85 GBq mmol^{-1}), [8-^{14}C]inosine (specific activity 1.92 GBq mmol^{-1}), [8-^{14}C]xanthosine (specific activity 2.07 GBq mmol^{-1}), [8-^{14}C]adenine (specific activity 1.85 GBq mmol^{-1}), [8-^{14}C]guanine (specific activity 1.96 GBq mmol^{-1}), [8-^{14}C]hypoxanthine (specific activity 1.85 GBq mmol^{-1}) and [8-^{14}C]xanthine (specific activity 1.85 GBq mmol^{-1}) from Moravek Biochemicals Inc. (Brea, CA, USA). Standard nucleotides, nucleosides and nucleobases, and biochemicals were purchased from Sigma-Aldrich, St. Louis, Mo, USA.

Administration of ^{14}C-labelled purine nucleosides and bases

Our experimental methods were essentially the same as Ashihara *et al.*[10] Incorporation of radioactivity into purine residues of nucleic acids was examined by the methods of Schmidt and Thannhauser[22] or Schneider,[23] with a slight modification as follows.[14] Suspension-cultured cells (~100 mg fresh weight) and 2 ml culture medium in which the cells had been grown were placed in the main compartment of a 30 ml Erlenmeyer flask. The flask was fitted with a glass tube containing a piece of filter paper impregnated with 0.1 ml of 20 % aq. KOH in a centre well, to collect $^{14}CO_2$. Each reaction was started by adding 10 µl (37 kBq) of [8-^{14}C]purine nucleoside or [8-^{14}C]purine base solution to the main compartment of the flask. The flasks were incubated in an oscillating water bath at 22 ºC.

Extraction of ^{14}C-labelled metabolites

After incubation, the glass tube was removed from the centre well and placed in a 50 ml-Erlenmeyer flask containing distilled water (10 ml). At the same time the cells were harvested by filtration over Miracloth (Calbiochem, La Jolla, CA, USA), washed with distilled water, and frozen with liquid N_2. They were then stored at -80 ºC. $KHCO_3$ that had been absorbed by the filter paper was allowed to diffuse into distilled water overnight, and aliquots of the resulting solution (usually 0.5 ml) were used for the determination of radioactivity.

The frozen cells were extracted with cold 6 % perchloric acid (PCA), using a glass homogenizer. After extraction, the homogenate was centrifuged at 12,000 *g* for 7 min. The resulting supernatant was collected and the precipitate was re-suspended with the same extraction reagent, and the supernatant was collected by centrifuging.

The first and second PCA-soluble fractions were combined and neutralized with 20 % aq. KOH. After brief centrifuging to remove potassium perchlorate, the samples were lyophilized overnight. For analysis of nucleic acids, the PCA-insoluble materials were extracted successively with a mixture of ethanol and diethylether (1:1, v/v) at 50 °C for 15 min. The ethanol and diethylether mixture insoluble fraction was hydrolysed with 0.3 M aq. KOH at 37 °C for 18 h, and adjusted to pH 2 with 6 M HCl. After centrifuging of the mixture at 10,000 *g* for 5 min, we collected the supernatant (RNA hydrolysates). DNA in the precipitate was hydrolysed with 6 % PCA at 100 °C for 20 min. Because the incorporation of radioactivity into the DNA fraction was negligible during the short incubation period (4 h), then, in order to simplify the methods, RNA and DNA were simultaneously hydrolysed with 6 % PCA at 100 °C for 20 min in some experiments.[23]

Analysis of ^{14}C-labelled metabolites

The PCA-soluble metabolites and hydrolysates of nucleic acids were neutralised, concentrated and loaded on microcrystalline cellulose TLC plates (Merck, Darmstadt, Germany) suitable for TLC analysis of ^{14}C-metabolites. The solvent systems used were (I) *n*-butanol–acetic acid–water (4:1:2, v/v/v) and (II) distilled water.[24,25]

The radioactivity of liquid samples was determined using a multi-purpose scintillation counter (Type LS 6500; Beckman, Fullerton, Calif., USA) with a liquid scintillation fluid ACS-II (GE Healthcare, Tokyo, Japan). Distribution of the radioactive spots of ^{14}C-metabolites on the TLC plate was determined using a Bio-Imaging Analyzer (FLA-2000, Fuji Photo Film Co. Ltd., Tokyo, Japan). Incorporation of radioactivity into individual metabolites was calculated from the total radioactivity of the liquid samples measured by the scintillation counter, and the % distribution of radioactivity on the plate obtained from the bio-imaging analyzer.

Determination of ATP and GTP

Nucleotides were extracted from *A. thaliana* cells (~500 mg fresh weight) and were analysed using an anion exchange column, Shim-pack WAX-1 (Shimadzu Corporation, Kyoto, Japan), as detailed in Ashihara et al.[26], with slight modifications. The freshly harvested cells were homogenized in chilled 6 % PCA with a glass homogeniser. The homogenates were centrifuged at 20,000 *g* for 20 min at 2 ºC, and the supernatant was collected and neutralized with 20 % aq. KOH. After brief centrifuging to remove potassium perchlorate, the samples were lyophilised. The dried samples were dissolved in the solvent for HPLC and filtered using disposable syringe filter units. Aliquots of 10–50 µl were taken for HPLC using a LC 10A HPLC system (Shimadzu Corporation, Kyoto, Japan). The absorbance at 260 nm was monitored using a Shimadzu Diode Array Detector, type SPD-M10A. Experiments to assess recovery were performed in parallel with all assays. To do this, known quantities of standard were added to the extraction medium in one member of each pair of duplicate samples prior to homogenisation. Recovery of standards usually exceeded 90 %. Some loss of ATP and GTP was observed when the cells were frozen with liquid nitrogen and stored in a deep-freezer at -80 ºC for a month. As a result, all assays were performed using freshly harvested cells and were completed on the same day.

Semi-quantitative RT-PCR

Total RNA was extracted from cells of *A. thaliana* at various stages of growth, as in our previous paper.[19] DNA-free total RNA was used for first strand cDNA synthesis. The reaction mixture (50 µl) contained 62.5 U of MuLv reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and 1 mM oligo-d(T)16. The PCR reaction mixture (25 µl) contained 40 ng cDNA and 12.5 µl GoTaq Green mastermix (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed according to the following program: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 7 min. The reaction product was visualised under UV light on 1 % agarose gels stained with SYBR Green I (Takara Bio Company, Tokyo, Japan), using a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan). The primers for *APT* and *ADK* were designed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi).The primers used in this study were as follows:

APT1 (GenBank accession no. At1g27450):
5′-ACCGTTCAACCACCTCACTC-3′ and
5′-AAAGGCCTCAGTGTCGAGAA-3′;

APT2 (At1g80050):
5′-GGGAGACCCGAGATTAAAGG-3′ and
5′-AACGAACTTGGCACCTATGG-3′;

APT3 (At4g22570):
5′-GATCCGTGTCGTTCCAGATT-3′ and
5′-ACGAGCCTCTATTCCTGCAA-3′;

APT4 (At4g12440):
5′-TCGTTCCAGATTTTCCCAAG-3′ and
5′-CCGGCAATTTCTTTGGTTTA-3′;

APT5 (At5g11160):
5′-TGTCGTTGCTGGAGTTGAAG-3′ and
5′-CAATGATAATGACGCGTTCG-3′;

ADK1 (At3g09820):
5′-GCCTGCCGTACATGGACTAT-3′ and
5′- AGCTTCTCTTTGGGGAGAGG-3′;

ADK2 (At5g03300):
5′-AAAACTGGGCATTGGTTGAG-3′ and
5′-GGCAAGAACTTCTCCTGCAC-3′;

Actin-2 (At3g18780):
5′-TGCCAATCTACGAGGGTTTC-3′ and
5′-TTCTCGATGGAAGAGCTGGT-3′.

Results

Profile of the proliferation of *A. thaliana* cells in suspension-culture

Growth of a suspension culture of *A. thaliana*, together with the concentration of chlorophyll during culture, is shown in Fig. 2. In general, following inoculation of suspension-cultured starved plant cells into the fresh culture medium, the cells undergo synchronous growth, as well as synchronous uptake and utilisation of the constituents of the medium.[1,27,28] The changing growth pattern of *A. thaliana* cells is essentially the same as that observed in culture cells of *Catharanthus roseus* (=*Vinca rosea*),[28] *Acer pseudoplatanus*,[29] *Datura innoxia*[30] and *Lotus japonicus*.[31]

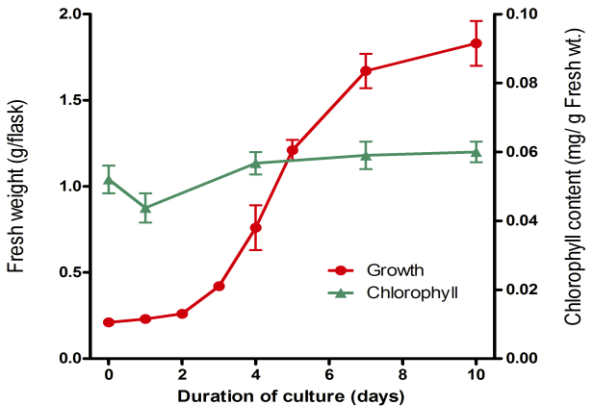

Figure 2. Fluctuations of fresh weight and chlorophyll content during growth of suspension-cultured *A. thaliana* cells. Fresh weight (g) is shown per culture, and the concentration of chlorophyll (mg) is expressed per g FW. Mean values and *SD* are shown.

When 10-day-old *A. thaliana* cells were transferred into the fresh culture medium, their fresh weight began to increase after a 2-day lag. Exponential growth began at day 3 and continued to day 7. After day 7, growth had reached the stationary phase. The chlorophyll concentration remained almost constant during culture, although a slight decrease was observed in the early lag phase of cell growth (Fig. 2).

In the present study, comparison of the metabolism of eight purine compounds was made using cells in the early-exponential phase (day 4). To determine whether the profile of the purine metabolism is changed during culture, we compared the metabolism of [8-^{14}C]adenine and [8-^{14}C]inosine in cells collected at days 0, 1, 4, 7, and 10. These cells are correspond to the initial-phase, the lag-phase, the cell division-phase, the cell expansion-phase and the stationary phase cells reported in the suspension culture of *C. roseus*.[28]

Uptake of [8-^{14}C]purine nucleosides and [8-^{14}C]purine bases by *A. thaliana* cells

Total uptake of precursors by the cells was calculated by adding the radioactivity found in PCA-soluble and PCA-insoluble fractions and CO_2. The purine nucleosides and bases were taken up by *A. thaliana* cells at differing rates during 4 h incubation time (Fig. 3).

Hypoxanthine was taken up fastest by the cells (84.0 nmol gFW^{-1}). Adenosine, guanosine, inosine, adenine and guanine were taken up at between 59 and 68 nmol gFW^{-1}. A relatively slow rate of uptake was found in xanthine (34 nmol gFW^{-1}) and xanthosine (41 nmol gFW^{-1}). The uptake rate was in the order hypoxanthine > adenine > inosine > guanosine > guanine > adenosine > xanthosine> xanthine (Fig. 3).

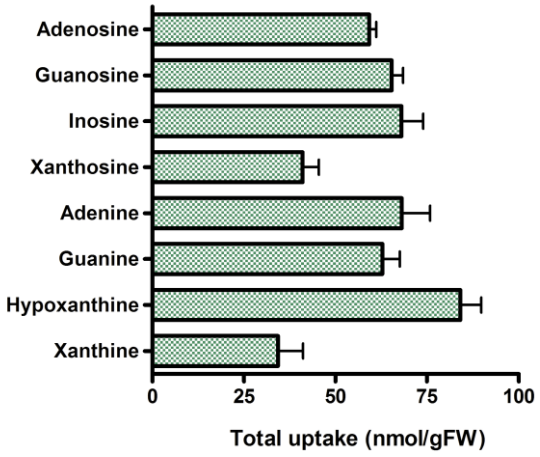

Figure 3. Uptake of [8-^{14}C]purine nucleosides and bases by 4-day-old cultured cells of *A. thaliana*. Incubation with labelled compounds took place for 4 h. The total uptake was calculated by summation of the radioactivity found in all cellular metabolites and CO_2. Uptake of purine compounds is expressed as nmol per g FW cells. Mean values and *SD* are shown.

***In situ* metabolism of [8-^{14}C]purine nucleosides**

To study the metabolic pathways of purine nucleosides in *A. thaliana*, the metabolic fate of [8-^{14}C]adenosine, [8-^{14}C]guanosine, [8-^{14}C]inosine and [8-^{14}C]xanthosine was investigated using 4-day-old *A. thaliana* cells (Fig. 4). After 4-h incubation the radioactivity from [8-^{14}C]nucleoside was found in salvage products (purine nucleotides and RNA), purine bases and/or catabolites (allantoin, allantoic acid, other unidentified catabolites and CO_2). Less than 20 % of the radioactivity was retained in unmetabolized ^{14}C-precursors.

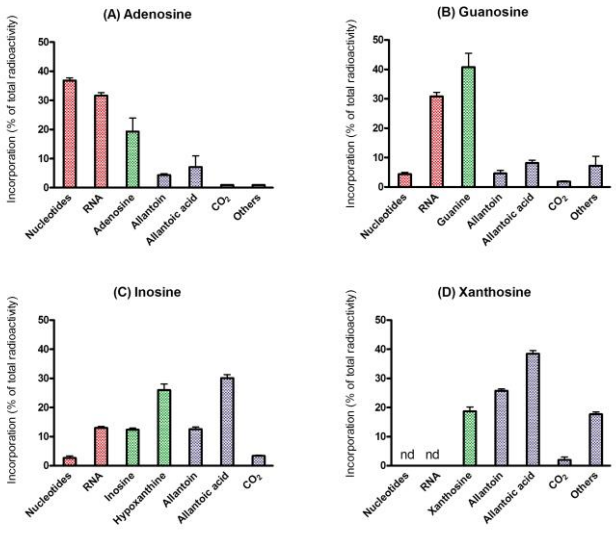

Figure 4. Metabolic fate of [8-^{14}C]adenosine (A), [8-^{14}C]guanosine (B), [8-^{14}C]inosine (C) and [8-^{14}C]xanthosine (D) in 4-day-old cultured cells of *A. thaliana*. The incubation time was 4 h. All detectable ^{14}C-metabolites are shown. The others are a few unidentified metabolites, possibly catabolites. The distributions of radioactivity are expressed as a percentage of the total radioactivity taken up by the samples ± *SD*. Red, green and blue bars respectively indicate salvage products, nucleoside and bases, and catabolites.

A. thaliana cells have different ability to form purine nucleotides from the four purine nucleosides. Radioactivity from [8-^{14}C]adenosine, [8-^{14}C]guanosine and [8-^{14}C]inosine was incorporated into the salvage compounds (nucleotides and RNA), but no incorporation from [8-^{14}C]xanthosine was observed (Fig. 4). Much of the radioactivity from [8-^{14}C]adenosine was incorporated into nucleotides (37 %) and RNA (32 %) (Fig. 4A). In RNA, the most radioactivity from [8-^{14}C]adenosine was recovered in adenine residues of RNA (74 %), and the rest was in guanine residues (26 %). These results suggest that adenosine is converted to AMP. A portion of AMP is converted to ATP or to GTP and then incorporated into RNA. Although 35 % of radioactivity from [8-^{14}C]guanosine was found in the salvage products, only 4 % was detected in nucleotides, and the rest (31 %) was found in guanine residues of RNA. This implies that guanosine is converted to GMP, and then GTP is formed via GDP. The smaller pool size of guanine nucleotides probably corresponds to weak distribution of ^{14}C in nucleotides. Conversion of GMP to adenine nucleotides appears to be negligible.

Only limited salvage ability of [8-^{14}C]inosine was observed. The radioactivity was found in nucleotides (3 %) and RNA (13 %) (Fig. 4C). In RNA, the radioactivity was distributed mainly in guanine residues (82 %), and the rest (18 %) was in adenine residues. In contrast to the three other purine nucleosides, neither nucleotides nor RNA were labelled when [8-^{14}C]xanthosine was administered to *A. thaliana* cells (Fig. 4D). This implies that the cells have no xanthosine salvage capability.

Considerable amounts of ^{14}C-labelled purine nucleosides and bases were found when cells were incubated with [^{14}C]nucleosides for 4 h (Fig. 4 A-D). In the case of [^{14}C]adenosine and [^{14}C]xanthosine, these nucleosides remained unaltered (Fig. 4A and 4D). [^{14}C]Guanosine was completely converted to guanine, (Fig. 4B) and some [^{14}C]inosine (Fig. 4C) was converted to hypoxanthine. This indicates high hydrolytic activity of guanosine and inosine in *A. thaliana* cells.

Radioactivity from the four [8-^{14}C]nucleosides was incorporated into allantoin, allantoic acid and CO_2. Small amounts of the radioactivity were also detected into other unidentified metabolites, possibly catabolites of allantoic acid (Fig. 4). For [8-^{14}C]adenosine and [8-^{14}C]guanosine, only limited amounts of radioactivity were recovered in these catabolites (Figs. 4A and 4B). In contrast, more than half of the total radioactivity from [8-^{14}C]inosine was found in hypoxanthine (26 %) and its catabolites, allantoin (13 %), allantoic acid (30 %) and CO_2 (3 %) (Fig. 4C). All radioactivity from [8-^{14}C]xanthosine metabolized by the cells was observed in catabolites. Nearly 65 % of the radioactivity was incorporated into ureides, specifically allantoin and allantoic acid (Fig. 4D). During the 4 h of the experiment, release of $^{14}CO_2$ from [8-^{14}C]purine nucleosides was only 1-3 % of the total radioactivity taken up by the cells.

In situ metabolism of [8-^{14}C]purine bases

We examined the metabolic fate of the four purine bases, [8-^{14}C]adenine, [8-^{14}C]guanine, [8-^{14}C]hypoxanthine and [8-^{14}C]xanthine in *A. thaliana* cells (Fig. 5). The metabolic profiles of these four purine bases are similar to those of the corresponding nucleosides (Fig. 4). Radioactivity from [8-^{14}C]adenine (58 %), [8-^{14}C]guanine (36 %) and [8-^{14}C]hypoxanthine (17 %) was incorporated into the salvage products (nucleotides and RNA), but no radioactivity was detected from [8-^{14}C]xanthine in the salvage products.

In RNA, the radioactivity from [8-^{14}C]adenine was mainly distributed in adenine residues (72 %), and the rest (28 %) was distributed in guanine residues, whereas radioactivity from [8-^{14}C]guanine and [8-^{14}C]hypoxanthine was extensively incorporated into guanine residues. This observation suggests that adenine is converted mainly to AMP, whereas guanine and hypoxanthine are used for the synthesis of GMP and IMP, respectively. As stated above, some AMP and GMP are converted to nucleoside triphosphate and are incorporated into RNA. No radioactivity from [8-^{14}C]hypoxanthine was recovered in the adenine residues of RNA. This implies that IMP derived from hypoxanthine is utilized preferentially in GMP synthesis, and conversion to AMP is very limited.

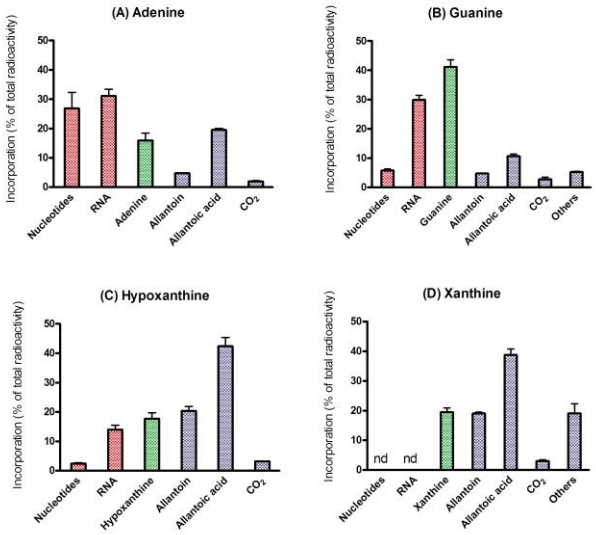

Figure 5. Metabolic fate of [8-^{14}C]adenine (A), [8-^{14}C]guanine (B), [8-^{14}C]hypoxanthine (C) and [8-^{14}C]xanthine (D) in 4-day-old cultured cells of *A. thaliana*. The incubation time was 4 h. All detectable ^{14}C-metabolites are shown. The others are a few unidentified metabolites, possibly catabolites. The distributions of radioactivity are expressed as a percentage of the total radioactivity taken up by the samples ± *SD*. Red, green and blue bars respectively indicate salvage products, nucleoside and bases, and catabolites.

All four purine bases were catabolised, and radioactivity was observed, in allantoin and allantoic acid, and in some cases in other catabolites (Fig. 5). Catabolic activity of hypoxanthine and xanthine was greater than that of adenine and guanine. Radioactivity was always higher in allantoic acid than in allantoin. The rate of $^{14}CO_2$ released from [^{14}C]purine bases during 4 h incubation corresponds to only 2-3 % of the total radioactivity.

Patterns in the metabolic fate of [8-^{14}C]adenine and [8-^{14}C]inosine during growth

[8-^{14}C]Adenine and [8-^{14}C]inosine have been used in several studies to determine the function of purine metabolism accompanied by growth and development of plant cells. They are suited to these studies because adenine metabolism is closely related to the status of the cellular energy metabolism and to the requirement of nucleic acid synthesis, and inosine metabolism is an indicator of the potential for catabolic activity of purine compounds.[32-35]

To study the profile of purine salvage and degradation during growth of *A. thaliana* cells, we monitored the metabolic fate of [8-^{14}C]adenine and [8-^{14}C]inosine 4 h after the labelled precursors had been administered to cells at different growth stages (Fig. 6). The greatest incorporation of radioactivity from [8-^{14}C]adenine into RNA was observed in cells in the lag phase (day 1), after which the rate of incorporation gradually decreased. The adenine residues of RNA are always heavily labelled (63-73 % of radioactivity recovered in RNA), more so than guanine residues (Fig. 7). In contrast, incorporation into nucleotides was high during the first 4 h after transfer of the stationary phase cells. Radioactivity in nucleotides decreased rapidly on day 1, and then increased gradually. The patterns of incorporation into

RNA and nucleotides were mirror images of each other. Incorporation of [8-^{14}C]adenine into ureides took place during the entire culture period, and radioactivity in allantoic acid (9.4–21.5 %) was always higher than in allantoin (2.3–6.7 %) (Fig. 6A).

RNA synthesis from [8-^{14}C]inosine is also observed. The highest incorporation was found in the cells at day 1 (Fig. 6B)**,** and 80-89 % of radioactivity in RNA was recovered in the guanine residues at every stage of growth (Fig. 7). Incorporation of radioactivity from [8-^{14}C]inosine into the nucleotides was extremely low (1.0-5.2 %), in contrast to [8-^{14}C]adenine. The highest rate of catabolism was observed in the first 4 h of inoculation, in which nearly 70 % of total radioactivity was recovered as catabolites.

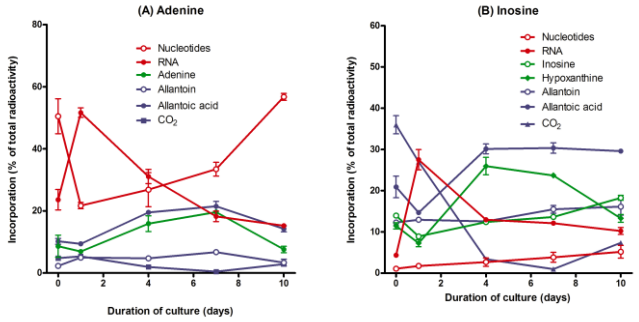

Figure 6. Metabolic fate of [8-^{14}C]adenine (A) and [8-^{14}C]inosine (B) in suspension cultured *A. thaliana* cells at distinct growth phases. Labelled compounds were administered to the 0, 1, 4, 7 and 10-day-old cells and incubated for 4 h. The distribution of radioactivity is expressed as % of total radioactivity taken up by the cells. All detectable ^{14}C-metabolites are shown. Mean values and *SD* are stated. Red, green and blue lines respectively indicate salvage products, nucleoside and bases, and catabolites.

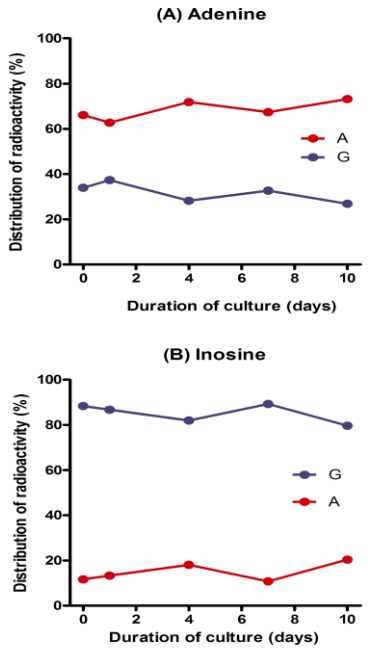

Figure 7. Incorporation of radioactivity from [8-^{14}C]adenine (A) and [8-^{14}C]inosine into the adenine and guanine residues of RNA in suspension-cultured *A. thaliana* cells at different growth phases. The distribution of radioactivity is expressed as % of radioactivity of RNA. Mean values and *SD* are shown.

Large amounts of $^{14}CO_2$ were released in the first two stages of culture, but this activity decreased rapidly 4 days after culture, coinciding with high radioactivity of allantoic acid (Fig. 6B).

Changes in ATP and GTP content during growth

The ATP and GTP content per culture flask increased at first 1 day after culture. A more prominent increase took place during days 4 to 7 (Fig. 8). The concentration of ATP and GTP expressed per g fresh weight increased markedly on the first day after transfer of the cells to the fresh medium (38.4 to 91.4 nmol gFW^{-1} for ATP and 9.3 to 22.1 nmol gFW^{-1} for GTP).

It decreased and remained almost constant (35.2-38.4 nmol gFW^{-1} for ATP and 7.3-11.4 nmol gFW^{-1} for GTP) until the end of the experiment (Fig. 8). The pool size of ATP was always 3-5 times larger than that of GTP. Since no visible increase in fresh weight was found on the first day of culture, there must be a net increase in ATP and GTP per cell. In *C. roseus* the highest concentration of ATP and GTP was also observed in the lag phase of cells.[36,37]

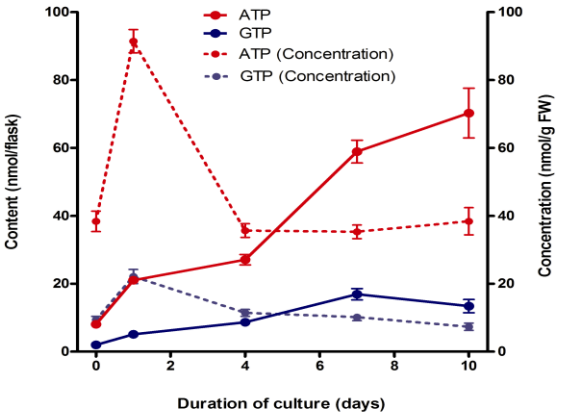

Figure 8. Fluctuation of the intracellular ATP and GTP during growth of *A. thaliana* cells in suspension culture. The nucleotide contents and concentrations are expressed as nmol flask^{-1} and nmol gFW^{-1}. Mean values and *SD* are shown.

Changes in levels of *APT* and *ADK* transcripts

The genome of *A. thaliana* contains five sequences specified as encoding adenine phosphoribosyltransferase, *APT1-APT5*. Adenine phosphoribosyltransferase catalyzes the conversion of adenine to AMP. This enzyme also catalyzes the formation of cytokinin riboside from cytokinin and PRPP. Allen *et al.*[38] have cloned *APT1*, *APT2* and *APT3*, and utilized over-expression in *E. coli* so as to compare kinetic properties for adenine and three cytokinin substrates (zeatin, isopentenyladenine, benzyladenine). The results suggest that *APT1* is involved in the conversion of adenine to AMP, while *APT2* and *APT3* are likely to participate in cytokinin interconversion. There is evidence for the expression of *APT1-APT*3, but no ESTs specific for *APT4* or *APT5* have been identified. Expression of *APT1* increased after cells were transferred to new medium, and the maximum expression was observed in the early-exponential phase (day 4), after which it decreased, whereas *APT 2* and *APT 3* were constitutive (Fig. 9).

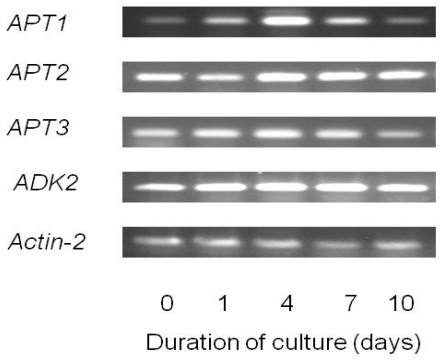

Figure 9. Expression of genes encoding adenine phosphoribosyltransferase (*APT1*, *APT2* and *APT3*) and adenosine kinase (*ADK2*) in suspension cultured *A. thaliana* cells, at different growth stages. Transcripts of *Actin-2* are also shown as standards.

The genes *ADK1* and *ADK2*, encoding two isoforms of adenosine kinase, have been isolated from *A. thaliana*.[39] In intact plants, *ADK1* expression was high in flowers, stems and roots, and *ADK2* expression was greatest in leaves.[39] In the present study using cultured cells, clear constitutive expression was observed only for *ADK2* (Fig. 9). For *ADK1* an ambiguous expression profile was detected (data not shown).

Discussion

Transport of purine nucleosides and bases

We have found that uptake of xanthosine and xanthine was slower than for other purine nucleosides and purine bases in several plant materials, including leaf disks of cacao,[16] slices of potato tubers,[14] and leaf and root segments of tea seedlings.[17] Although we have not carried out detailed kinetic studies of the uptake of these purine compounds here, our results from rather short-duration experiments clearly indicate the presence of substrate-specific transporters for certain nucleosides and bases in plants. Two types of nucleoside transporter, equilibrative and concentrative, have been postulated, but only equilibrative nucleoside transporters have been identified in *A. thaliana*.[40] Recently, Riegler *et al.*[6] reported that roots of *A. thaliana* seedlings took up [8-^{14}C]inosine and [8-^{14}C]xanthosine at almost the same rate (~57 nmol gFW^{-1}) after 48 h-incubation. It is possible that the discrepancy between Riegler's results and our own is due to the incubation time. Our results were obtained using the shorter incubation time during which uptake was linear throughout.

Possible salvage of purine nucleosides and bases in *A. thaliana*

From the *in situ* ^{14}C-tracer experiments (Figs. 4 and 5) and the information obtained from our previous enzymatic studies of potato tubers[14] and tea leaves,[17] pathways of purine salvage in *A. thaliana* can be proposed. Possible metabolic pathways are shown in Fig. 10. Adenosine is exclusively salvaged to AMP by adenosine kinase (EC 2.7.1.20, step 1), and guanosine and inosine are respectively converted to GMP and IMP by inosine/guanosine kinase (EC 2.7.1.73, step 2). Non-specific nucleoside phosphotransferase (EC 2.7.1.77, step 14) may also be involved in these reactions in plant cells.[14]

In situ inosine salvage activity was less than for guanosine salvage (Fig. 4B, 4C), although the step is catalyzed by the same enzyme, inosine/guanosine kinase (EC 2.7.1.73, step 2). Since this enzyme catalyzes the formation of nucleotides from inosine and guanosine at a similar rate,[14] the lesser inosine salvage may be due to the high catabolic activity of inosine.

Unlike the other purine nucleosides, xanthosine salvage is not evident in our ^{14}C-tracer experiments (Fig. 4). No xanthosine kinase has been detected in plants, and plant (non-specific) nucleoside phosphotransferase (EC 2.7.1.77) cannot use xanthosine as a substrate.[14] Riegler *et al.*[6] reported active xanthosine salvage for RNA synthesis in intact seedlings of *A. thaliana*, however, in which 14 % of the total radioactivity from [8-^{14}C]xanthosine was recovered in the RNA fraction. The results of Riegler *et al.*[6] also differed from our previous results using disks of potato tubers[14] and leaf disks of cacao[16] and tea.[17] Riegler *et al.*[6] suggested that the discrepancy between our results and theirs arose from the difference in experimental conditions; they used intact seedlings which more closely resembled natural situations, whereas we used artificial excised tissues. To see whether our previous results using excised tissues reflect purine metabolism *in planta*, we therefore studied (in an earlier paper[41]) the metabolism of [8-^{14}C]xanthosine in intact mungbean seedlings. We found no significant salvage of xanthosine to RNA, even in intact plants. We therefore supposed that low xanthosine salvage in plants is probably due to the different plant species used, and almost certainly not due to the excision of the tissues. The present results indicate that xanthosine salvage does not occur in *A. thaliana* cells, however. Presumably the discrepancy is due to differences in experimental procedure. As described elsewhere,[41] the RNA used by Riegler *et al.* may include some contaminants; they did not confirm the radioactivity in the purine residues of RNA.[6] Slow salvage and rapid catabolism of xanthosine appear to be inherent properties of many plant species.

Of the purine bases, adenine, guanine and hypoxanthine are salvaged by adenine phosphoribosyltransferase (EC 2.4.2.7, step 3), and by hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8, step 4). These purine bases are usually not salvaged by purine nucleoside phosphorylase (EC 2.4.2.1, step 18), as this enzyme was not detected in many other plant materials.[1,14] In the present study, we detected no xanthine salvage in *A. thaliana* cells (Fig. 5D). No activity of xanthine phosphoribosyltransferase is detectable in potato tuber extracts,[14] but low levels of activity have recently been found in tea leaf extracts.[17] Activity of guanine/xanthine phosphoribosyltransferase and hypoxanthine/guanine/xanthine phosphoribosyltransferase has also been reported in *Escherichia coli* [42] and in some parasites.[43,44] Theoretically, therefore, xanthine salvage may be possible. However, ^{14}C-tracer experiments strongly suggest that xanthine is catabolised predominantly by the oxidative purine ring catabolic pathway, and that virtually no xanthine salvage for XMP synthesis occurs in *A. thaliana*.

Possible catabolic pathways of purines

Our tracer experiments suggest that an oxidative purine degradation pathway is active in *A. thaliana* cells **(**Figs. 4 and 5**)**. As Fig. 10 shows, the first distinct step in purine

catabolism is the xanthine dehydrogenase reaction (EC 1.1.1.204, step 20). There are several routes for producing xanthine from other purine compounds in plants. Exogenously supplied adenosine and adenine are not directly deaminated to inosine and hypoxanthine, because plant cells contain neither adenosine deaminase (step 16) nor adenine deaminase (step 19).[1] Consequently adenosine and adenine are first salvaged to AMP and then converted to xanthine via IMP (steps 12, 13, 15 and 20) or via XMP (steps 12, 10, 13, 15). Deamination of the adenine structure is performed by AMP deaminase (EC 3.5.4.6, step 12). In contrast, inosine and xanthosine are hydrolyzed to their respective purine bases by inosine/guanosine nucleosidase (EC 3.2.2.2, step 15). Guanosine is deaminated to xanthosine by guanosine deaminase (EC 3.5.4.15, step 17), and xanthosine is hydrolyzed to xanthine (15). Guanine deaminase (EC 3.5.4.3, step 21) has been found in tea leaves,[45] but was not detected in potato tubers.[14] Recent molecular biological studies indicate that guanosine deaminase is required for the generation of xanthosine in *A. thaliana*.[46] At least in *A. thaliana*, catabolism of guanine is therefore performed after guanine has been salvaged to GMP (step 4). A pathway GMP → guanosine → xanthosine → xanthine (steps, 13, 17, 15) is the major path for guanine nucleotide catabolism in *A. thaliana*.[46]

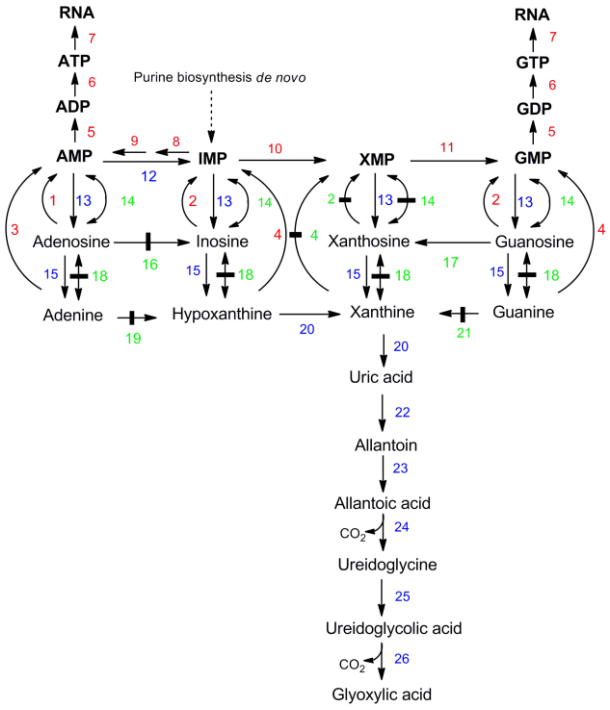

Figure 10. Metabolic pathways of purine compounds in *A. thaliana* cells estimated from the fate of exogenously supplied [8-^{14}C]purine nucleosides and [8-^{14}C]purine bases. The numbers show the enzymes participating in individual reactions. Arrows broken with a vertical bar represent reactions which are not present in *A. thaliana*. Enzymes: 1, adenosine kinase; 2, inosine/guanosine kinase; 3, adenine phosphoribosyltransferase; 4, hypoxanthine/guanine phosphoribosyltransferase; 5, nucleoside monophosphate kinase; 6, nucleoside diphosphate kinase; 7, RNA polymerase; 8, adenylosuccinate synthetase; 9, adenylosuccinate lyase; 10, IMP dehydrogenase;11, GMP synthetase; 12, AMP deaminase; 13, 5'-nucleotidase; 14, nucleoside phosphotransferase; 15, adenosine nucleosidase and/or inosine-guanosine nucleosidase; 16, adenosine deaminase; 17, guanosine deaminase; 18, purine nucleoside phosphorylase; 19, adenine deaminase; 20, xanthine dehydrogenase; 21, guanine deaminase; 22, allantoin synthase; 23, allantoin amidohydrolase; 24, allantoate amidohydrolase; 25, ureidoglycine aminohydrolase; 26, ureidoglycolate amidohydrolase.

In contrast to adenine and guanine, hypoxanthine and xanthine are intermediates of the purine catabolic pathway, and these compounds are therefore more readily catabolized (Fig. 5). These results indicate that release of the amino group in the purine ring at the AMP deaminase or guanosine deaminase steps is important in determining whether purine molecules are salvaged or catabolized.

Catabolism of the purine ring

In the present study, radioactivity from [8-^{14}C]purine nucleosides and purine bases was found to be incorporated into allantoin, allantoic acid and CO_2. The highest radioactivity was found in allantoic acid. Our results suggest that the purine ring is easily catabolised to allantoic acid via allantoin (steps 20 and 22). Details of ureide catabolism have recently been determined.[47-49] In *A. thaliana*, the pathways catalysed by allantoinase (EC 3.5.2.5, step 23), allantoate amidohydrolase (EC 3.5.3.9, step 24), ureidoglycine aminohydrolase (EC 3.5.3.26, step 25) and ureidoglycolate amidohydrolase (EC 3.5.1.116, step 26), have been proposed as active in the complete hydrolysis of allantoin. We did not find the details of the catabolic pathway, because we did not detect radioactivity in intermediates of the pathway. If this pathway is operative, the 8 position carbon of the purine ring is released as CO_2 at the final stage (step 26) of purine catabolism.

Purine metabolism during proliferation of *A. thaliana*

Using *A. thaliana* cells in the early-exponential phase (day 4), we examined the metabolic fate of purine nucleosides and bases. To determine whether the patterns of metabolism are similar in cells in other stages, we compared the pattern of metabolism of selected purine compounds. Salvage of adenine is active in every stage of culture. In cells in the initial phase (0-4 h), nucleotides were more heavily labelled than in the following lag phase (day 1) in which radioactivity shifting into RNA was maximum. This suggests that ATP accumulation is preceded by RNA synthesis. Accumulation of ATP by the salvage pathway, as well as conversion of AMP and ADP to ATP, may begin immediately after cell transfer to the fresh medium, and accumulation of ATP may act as a trigger of nucleic acid and protein synthesis, as observed in other cultured system.[50] A similar pattern was also found in RNA synthesis from [8-^{14}C]inosine. This suggests that purine salvage contributes to the initial RNA synthesis which begins immediately after the cells are transferred to the fresh medium; this may initiate the following cell division.

Catabolic activity of inosine is higher than that of adenine during every culture phases. Marked release of $^{14}CO_2$ from [8-^{14}C]inosine was observed in the initial and lag culture phases. This suggests that degradation of allantoic acid is extremely active in the early stages of cell proliferation. In the allantoin degradation, glyoxylic acid and ammonia are produced as end products, together with CO_2. The *A. thaliana* cells are able to recycle these end products to synthesize new organic molecules for use in proliferation.[48] The degradation of allantoin in the early phase of proliferation may be for this purpose.

The metabolic fate of exogenously supplied [^{14}C]precursors in various stages of the growth of *A. thaliana* cells shows the ability of purine salvage and purine catabolism in the cells of corresponding proliferation stages. The results are influenced by the concentration of endogenous precursors and intermediates. Furthermore, *in planta* purine metabolism is dependent upon the endogenous supply of precursors. The net increase of purine nucleotides accompanying cell proliferation requires the *de novo* purine biosynthesis, because the supply of purine nucleosides and bases for salvage pathways is limited. We confirmed the activity of the *de novo* purine biosynthesis using [^{14}C]formate and [2-^{14}C]glycine in *A. thaliana* (data not shown). Estimation of the relative activity of the *de novo* pathway during cell proliferation is difficult, because [^{14}C]formate and [2-^{14}C]glycine are utilised not only for nucleotide biosynthesis but also in many other metabolic pathways.

Menges *et al.*[51] reported genome-wide gene expression in suspension cultured cells of *A. thaliana*. They presented an integrated genome-wide view of the transcriptional profile of a plant suspension culture, and identified a refined set of 1082 cell cycle regulated genes. Neither *APT* nor *ADK* genes appeared in their lists, however. Our results suggest that *APT1* and *ADK2*, which encode the enzymes for AMP synthesis from adenine and from adenosine, are expressed differently.

Adenine phosphoribosyltransferase requires 5-phosphoribosyl-1-pyrophosphate (PRPP) for reaction. Our previous paper reported the expression of the *PRS* genes encoding phosphoribosylpyrophosphate synthetase.[18] As well as the generally distributed phosphate-dependent PRPP synthetase (class I), phosphate-independent PRPP synthetase (class II) is also present in plants. The transcript levels of *PRS1* and *PRS2* encoding class I enzymes and *PRS3* encoding class II enzymes increased rapidly after the cells were transferred to the fresh medium, and then remained almost constant during the early exponential growth phase. Constitutive expression of *PRS4* encoding cytosolic class II enzyme was observed during culture. The changing profile of transcript levels of *APT1* is similar to *PRS1-3*, and it follows that the adenine salvage system may be induced in the early phase of cell proliferation. The adenosine salvage system, in contrast, is somewhat constitutive.

The endogenous pool size of nucleosides and free nucleobases is usually small in plant cells. In *C. roseus* cells, for example, the pool of adenosine varied from 2 to 9 nmol gFW^{-1} during culture, and free adenine was practically undetectable.[52] This fact suggests that the salvage activity of adenine and adenosine is very high, and that nucleosides and bases generated by the degradation of nucleotides are immediately salvaged to nucleotides *in planta*.

Conclusion

Using ^{14}C-labelled purine nucleosides and bases, the present study found very clearly that adenosine, guanosine, adenine and guanine are salvaged to nucleotides and utilized for RNA synthesis. Inosine and hypoxanthine are also salvaged to nucleotides, mainly guanine nucleotides. These compounds are intermediates of the purine nucleotide catabolism, and so significant amounts are converted to xanthine and enter the catabolic pathway via allantoin. Neither xanthosine nor xanthine is used for the synthesis of nucleotides and RNA. These compounds are catabolized via allantoin and allantoic acid. The results indicate that release of the amino group in the purine ring is a crucial step in determining whether purine molecules are salvaged or catabolized. Deamination of adenine and guanine rings proceeds at the stage of AMP and guanosine, respectively. The pattern of purine metabolism in *A. thaliana* is similar to that in other plants.

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