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Effect of long-term phosphate starvation on the levels and metabolism of purine nucleotides in suspension-cultured *Catharanthus roseus* cells

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Abstract

The effect of long-term phosphate (Pi) starvation of up to 3 weeks on the levels of purine nucleotides and related compounds was examined using suspension-cultured *Catharanthus roseus* cells. Levels of adenine and guanine nucleotides, especially ATP and GTP, were markedly reduced during Pi-starvation. There was an increase in the activity of RNase, DNase, 5′- and 3′-nucleotidases and acid phosphatase, which may participate in the hydrolysis of nucleic acids and nucleotides. Accumulation of adenosine, adenine, guanosine and guanine was observed during the long-term Pi starvation. Long-term Pi starvation markedly depressed the flux of transport of exogenously supplied [8-^{14}C]adenosine and [8-^{14}C]adenine, but these labelled compounds which were taken up by the cells were readily converted to adenine nucleotides even in Pi-starved cells, in which RNA synthesis from these precursors was significantly reduced. The activities of adenosine kinase, adenine phosphoribosyltransferase and adenosine nucleosidase were maintained at a high level in long-term Pi starved cells.

Keywords: *Catharanthus roseus*; Apocynaceae; Madagascar periwinkle; Suspension culture; Phosphate starvation; Purine nucleotides

1. Introduction

Plants exhibit morphological and molecular adaptations to Pi deprivation. Recently, changes in gene expression during Pi starvation were investigated systematically using ''smart'' plants that monitor plant phosphorus status in *Arabidopsis* (Hammond et al., 2003). In the resulting screening of specifically expressed genes after Pi-starvation, the only recorded gene that was related to the nucleotide metabolism was the gene encoding RNase. However, it has been suggested that the transcription of genes of RNases and phosphatases is accelerated by Pi-starvation and is followed by an increase in the activity of enzymes that hydrolyse nucleic acids and nucleotides (Abel et al., 2000; Baldwin et al., 2001; Bariola et al., 1994; Duff et al., 1991, 1994; Goldstein et al., 1988; Li et al., 2002; Löfler et al., 1992). Induction of these hydrolysing enzymes may cause dramatic changes in the metabolism of phosphorylated compounds. However, in contrast with molecular studies of these enzymes themselves, only limited investigations have been performed on changes in the levels of phosphorylated metabolites and related metabolism during Pi-starvation. This approach at the cellular level is also important in understanding the biological phenomena caused by Pi deficiency (Stasolla et al., 2003; Wagner and Backer, 1992). In cultured *Catharanthus roseus* cells, we have found that levels of sugar phosphates, nucleotides, RNA and proteins are decreased, but levels of free amino acids and phenolic compounds are increased during

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short-term Pi-starvation (Ashihara and Ukaji, 1986; Li and Ashihara, 1989, 1990; Ukaji and Ashihara, 1986, 1987a,b). These studies were performed using an experimental system of short-term Pi-starvation varying from a few hours to a few days. In the present study, we investigated the effect of long-term phosphate starvation for up to 3 weeks on levels of purine nucleotides, nucleosides and nucleobases (Fig. 1), since purine nucleotides are closely related to the energy status of the cells. Our results indicate that the levels of adenine (**1**) and guanine nucleotides are greatly influenced by Pi-starvation. Long-term Pi starvation caused an increase in the activity of some hydrolysing enzymes, and led to accumulation of purine nucleosides and bases in the cells. Transport of adenosine (**2**) and adenine (**1**) across the cell membrane was markedly reduced by long-term Pi-starvation, but the machinery of salvage of purine nucleosides and bases was maintained. We determined the changing profiles of purine nucleotides, nucleosides and nucleobases in response to Pi-starvation using two different HPLC systems, and examined the activity of several enzymes that are closely related to nucleotide degradation and synthesis. Based on these results, the physiological significance of purine degradation and salvage in Pi-starved cells are discussed.

2. Results and discussion

2.1. Establishment of long-term Pi-starved culture system

A synchronous cell division system of *C. roseus* has been established using the phosphate starvation method (Amino et al., 1983). We used a similar culture system for this study, because the growth of the *C. roseus* cell cultures shows a clear dependence on Pi. The stationary phase (7-day-old) cells of a stock culture of *C. roseus* were transferred to fresh MS medium containing 1.25 mM Pi (+Pi medium) for 7 days. The numbers of cells per culture flask and the cellular Pi contents per 10^7 cells are shown in Figs. 2(a) and (b). The cell numbers increased after cell transfer, and reached the stationary phase at day 7, when Pi and other nutrients in the medium were almost exhausted. The highest cellular Pi concentration was observed on day 1, after which most Pi was converted to organic phosphates, such as sugar phosphates, nucleotides, nucleic acids and phospholipids (Ashihara and Tokoro, 1985). To obtain long-term Pi-deficient cells, the 7-day-old cells grown in the MS medium ("+Pi medium") were transferred to the fresh Pi-free MS medium ("−Pi medium"), and were cultured for three more weeks (day 7 to day 28 in Fig. 2). Little or no increase in cell numbers was observed (Fig. 2(a)), and the cellular Pi level was maintained at a low level (Fig. 2(b)). Cell numbers increased when Pi was added to the culture, which had been maintained in the Pi-free medium for 3 weeks (data not shown). It appears that *C. roseus* cells may be resting during Pi starvation, and resume growth when Pi is supplied. The

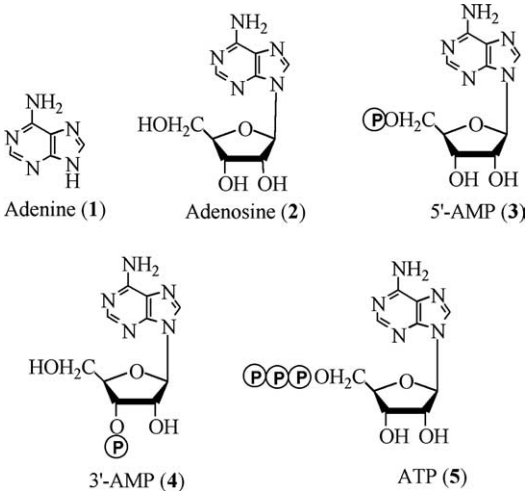

Fig. 1. Structure of the adenine, adenosine, 5′-AMP, 3′-AMP and ATP.

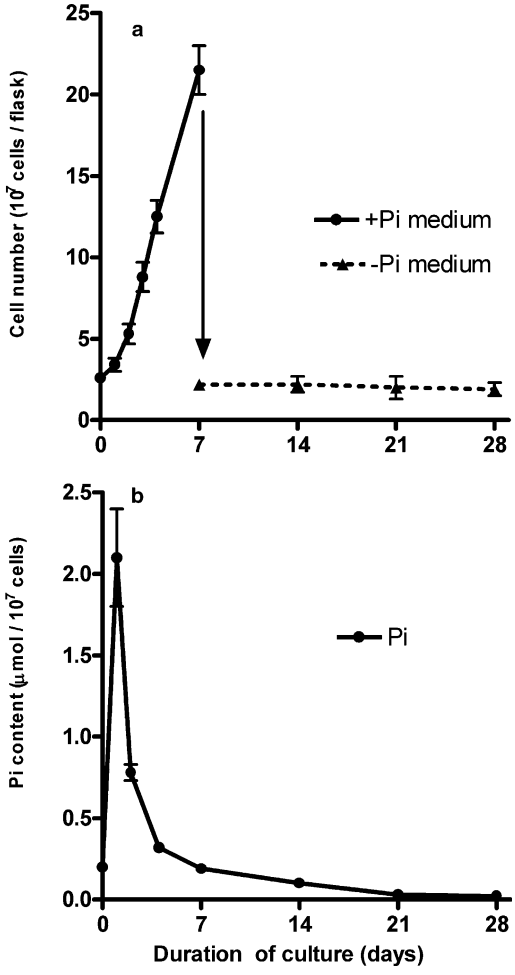

Fig. 2. Experimental system for long-term Pi-starvation of *Catharanthus roseus* cells. After a cycle of growth with ordinary MS ("+Pi") medium containing 1.25 mM Pi (days 1–7), the cell suspension was transferred to Pi-free fresh MS ("−Pi") medium and was cultured for 3 weeks. Samples were collected weekly (days 14, 21 and 28). (a) Changes in cell numbers expressed as 10^7 cells per a culture flask; (b) cellular level of Pi expressed in μmol per 10^7 cells.

present study used the 4-week experimental system shown in Fig. 2. Sample cells were collected at days 1 and 7 from the initial Pi-fed culture. The long-term Pi-starved cells were obtained from culture in the "−Pi medium". One to three-week-old cells in the "−Pi medium" correspond to the cells at days 14, 21 and 28 in Figs. 3–9.

2.2. Changes in contents of RNA and purine nucleotides during Pi-starvation

The RNA content in the cells at days 1, 7, 14, 21 and 28 was, respectively, 267 ± 55, 172 ± 45, 154 ± 25, 121 ± 33 and 133 ± 17 μg per 10^7 cells.

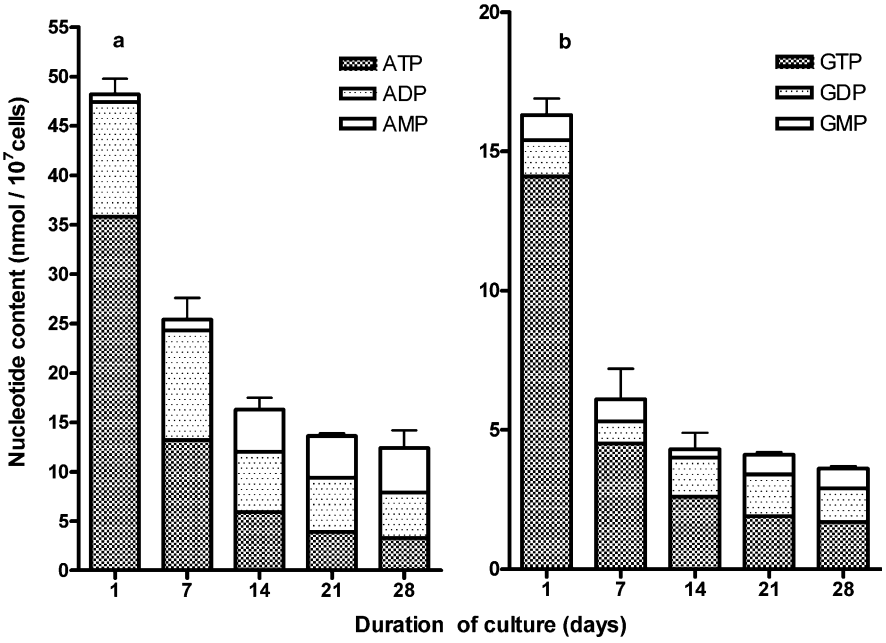

Fig. 3. Changes in purine nucleotides in *Catharanthus roseus* cells during, before and after Pi starvation. Experimental system, see Fig. 1. Nucleotide content is expressed as nmol per 10^7 cells. Standard deviations for total content are also shown ($n = 4$). (a) Adenine nucleotides (ATP (5), ADP and AMP); (b) guanine nucleotides (GTP, GDP and GMP).

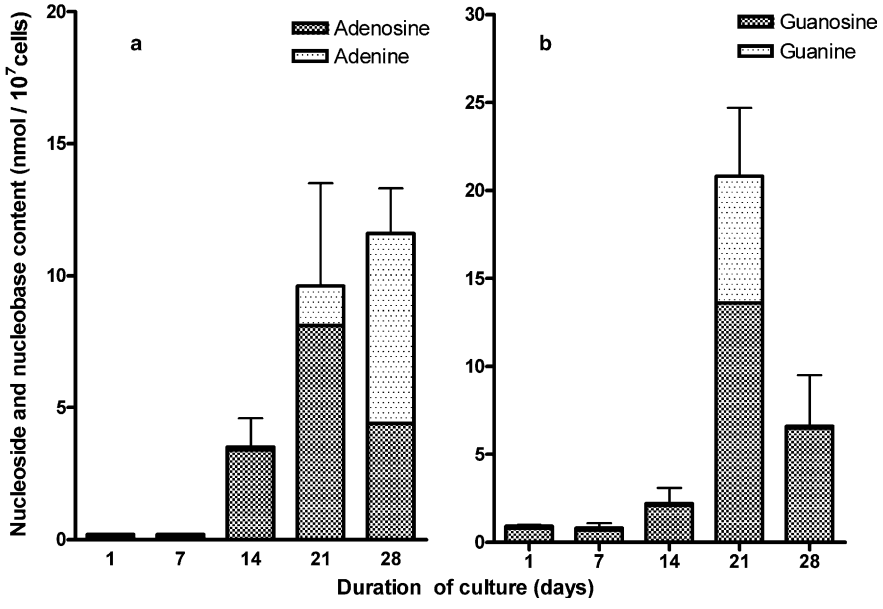

Fig. 4. Changes in purine nucleosides and bases in *Catharanthus roseus* cells during, before and after Pi starvation. Experimental system: see Fig. 1. Nucleoside and nucleobase content is expressed as nmol per 10^7 cells. Standard deviations for total content are also shown ($n = 4$). (a) Adenosine (**2**) and adenine (**1**); (b) guanosine and guanine.

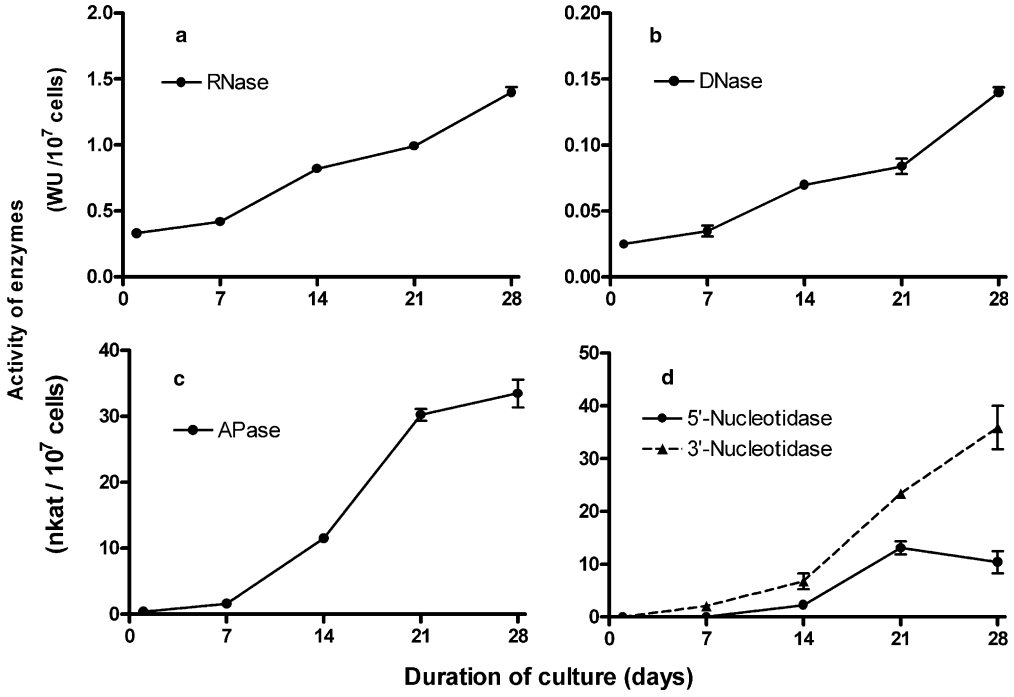

Fig. 5. Changes in activity of hydrolysing enzymes during culture of *Catharanthus roseus* cells during, before and after Pi starvation. Experimental system: see Fig. 1. Enzyme activity is expressed as Wilson unit (a and b) or nkat (c and d) per 10^7 cells ± SD($n = 3$). (a) RNase; (b) DNase; (c) acid phosphatase; (d) 5′-nucleotidase (circle) and 3′-nucleotidase (triangle).

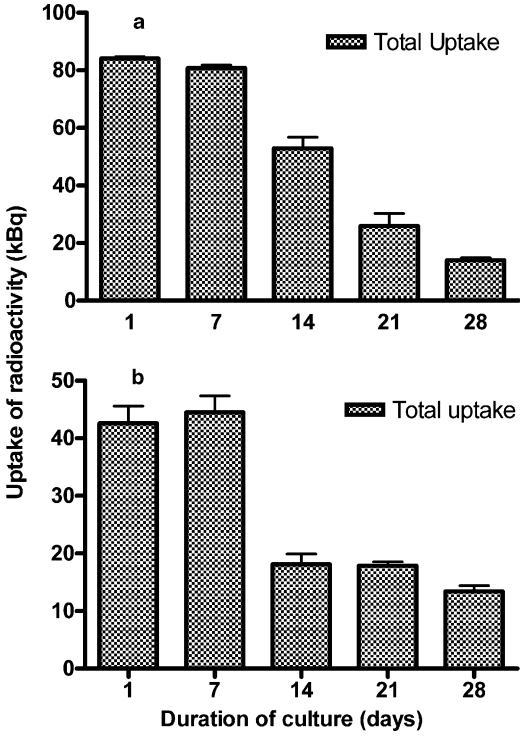

Fig. 6. Changes in the uptake of [8-^{14}C]adenosine (**2**) (a) and [8-^{14}C]adenine (**1**) (b) by *Catharanthus roseus* cells. Experimental system: see Fig. 1. Total uptake of radioactivity during 3 h incubation is expressed as kBq per 10^7 cells ± SD ($n = 3$).

Fig. 3 shows the levels of adenine nucleotides (a) and guanine nucleotides (b), expressed as nmol per 10^7 cells. One day after the cells were transferred to fresh +Pi medium, the maximum cellular levels of free adenine nucleotides and guanine nucleotides were observed. The major components of these nucleotides were ATP (5) (35.8 nmol) and GTP (14.1 nmol). The adenylate energy charge (Atkinson, 1977) calculated from the data was 0.86. Total purine nucleotide levels decreased significantly at day 7. After the 7-day-old cells were transferred to the "−Pi medium", a marked decrease in ATP (**5**) and GTP took place. The ratio of nucleoside di- and monophosphates to nucleotide triphosphates increased in Pi-starved cells. The lowest adenylate energy charge (0.45) was obtained from 28-day-old cells, in which the AMP level was higher than that of ATP (5). Other purine nucleotides, such as inosine-5′-monophosphate and xanthosine-5′-monophosphate, could not be detected in the cells at any experimental time.

2.3. Changes in purine nucleosides and bases during Pi-starvation

The levels of purine nucleosides and bases are shown in Fig. 4. Very low amounts of nucleosides and bases were detected in cells cultured in the "+Pi medium" (days 1 and 7). After cells were transferred to the "−Pi medium", accumulation of these compounds was observed. Initially, adenosine (**2**) was accumulated (day 14), but after longer Pi-starvation (day 28) adenine (**1**) became a major component. A similar trend was found in guanosine and guanine, but guanine had disappeared after 3 weeks of Pi-starvation (day 28). Compared with adenine nucleotides, the contents of adenosine (**2**) and adenine (**1**) were even lower in long-term Pi-starvation, but the guanosine content at days 21

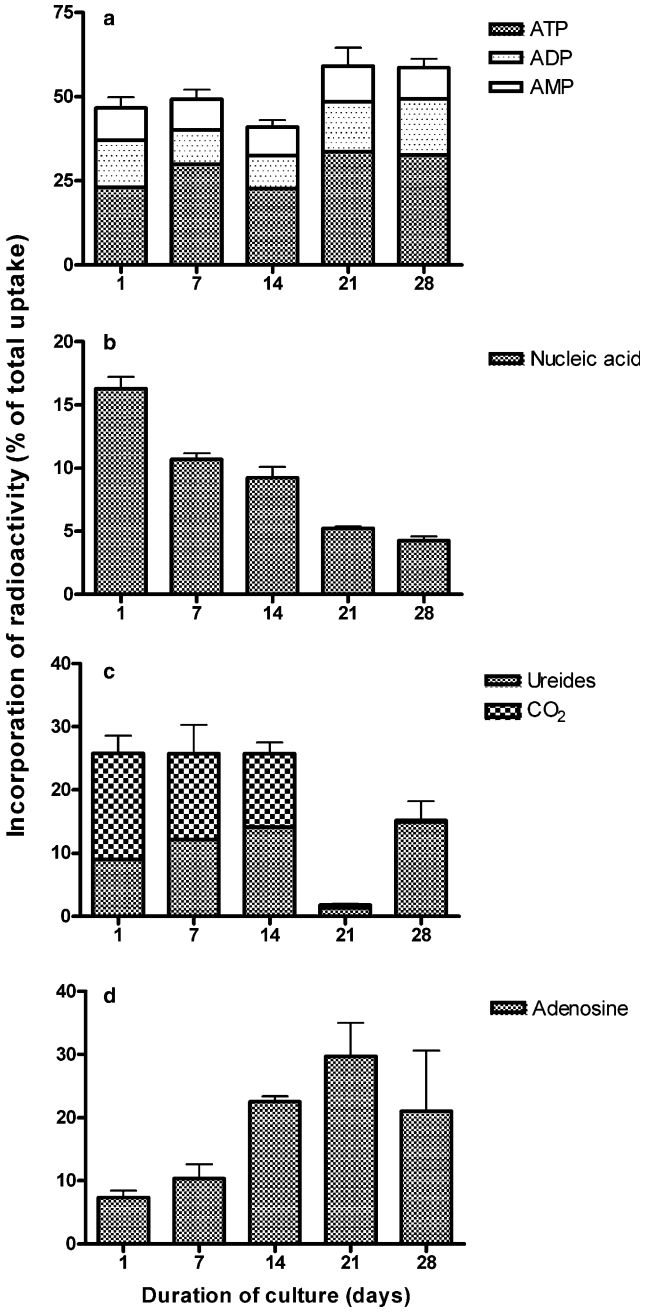

Fig. 7. Distribution of radioactivity from [8-^{14}C]adenosine (**2**) into nucleotides (a), nucleic acid (b), degradation products (c) and adenosine (**2**) (d). Values are expressed as a percentage of total uptake. Standard deviations for total values are also shown ($n = 3$). Ureides consist of allantoin and allantoic acid. Small amounts of radioactivity (<1%) distributed in adenine (**1**) and some other compounds are not listed.

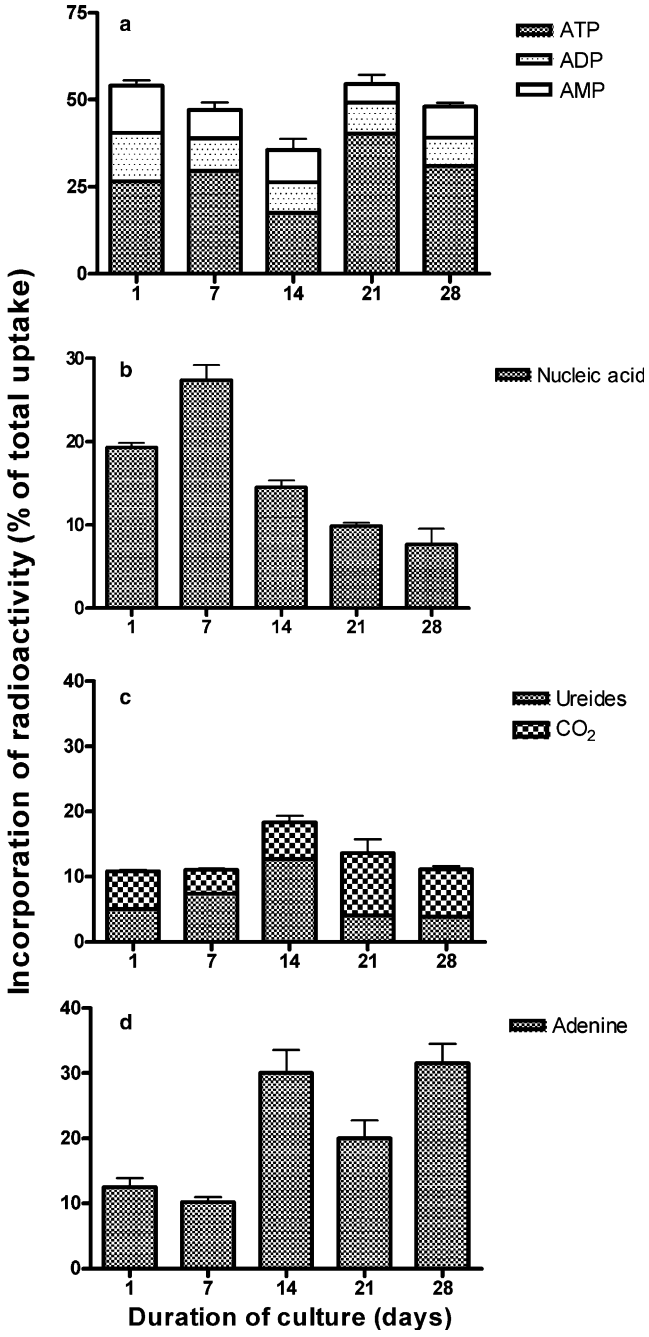

Fig. 8. Distribution of radioactivity from [8-^{14}C]adenine (**1**) into nucleotides (a), nucleic acid (b), degradation products (c) and adenine (1) (d). Values are expressed as a percentage of total uptake. Standard deviations for total values are also shown ($n = 3$). Small amounts of radioactivity (<1%) distributed in adenosine (**2**) and other compounds are not listed.

and 28 was higher than that of guanine nucleotides. No accumulation of inosine, xanthosine, hypoxanthine, xanthine or uric acid was found.

2.4. Changes in activity of hydrolases during Pi-starvation

Fig. 5 shows changes in activity of RNase, DNase, acid phosphatase, 5′-nucleotidase and 3′-nucleotidase measured in crude enzyme preparation. As suggested by many investigators (Raghothama, 1999), RNase, DNase and acid phosphatase activities increased markedly during Pi-starvation. A preliminary study indicated that the order of preferred substrate of both enzymes was AMP > GMP > CMP > UMP (data not shown). We therefore chose 5′-AMP (**3**) and 3′-AMP (**4**) as substrates. The activity of 3′-nucleotidase was always higher than that of 5′-nucleotidase. As with other hydrolytic enzymes, a high activity of 5′-nucleotidase and 3′-nucleotidase was found in long-

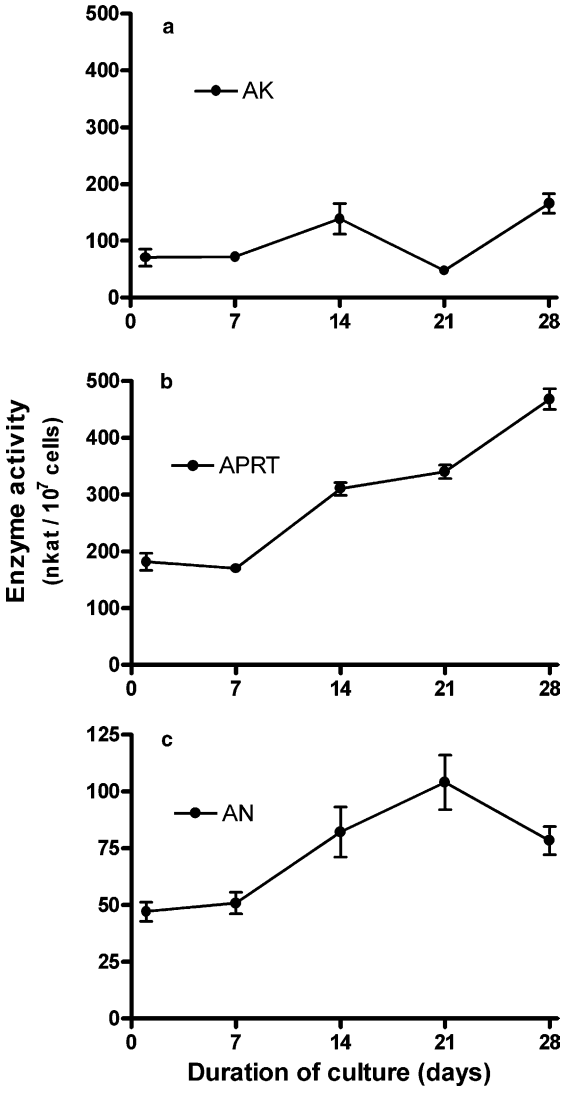

Fig. 9. Changes in activity of enzymes related to purine salvage during culture of *Catharanthus roseus* cells. Experimental system: see Fig. 2. Enzyme activity is expressed as nkat per 10^7 cells ± SD ($n = 3$). (a) adenosine kinase; (b) adenine phosphoribosyltransferase; (c) adenosine nucleosidase.

term Pi-starved cells. The decrease in nucleotides during Pi starvation seems to be substantially correlated with the increase in 5′-nucleotidase activity.

2.5. Changes in metabolism of adenosine (2) and adenine (1) during Pi-starvation

Adenosine (**2**) and adenine (**1**) are not intermediates of de novo adenine nucleotide synthesis, but are produced as the degradation products of nucleotides, *S*-adenosyl-L-methionine and nucleic acids (Stasolla et al., 2003). It has been well known that these compounds are re-utilised for the nucleotide and nucleic acid synthesis by so-called salvage pathway (Stasolla et al., 2003). Adenosine (**2**) and adenine (**1**) were not degraded directly, but after conversion to adenine nucleotides they are degraded to NH_3 and CO_2 by a conventional purine catabolic pathway via allantoin (Yabuki and Ashihara, 1991). We investigated whether the phosphate status of the cells affects the transport and metabolism of adenosine (**2**) and adenine (**1**). For this purpose, we monitored the uptake and metabolic fate of exogenously supplied [8-^{14}C]adenosine (**2**) and [8-^{14}C]adenine (**1**) in *C. roseus* cells cultured in "+Pi" and "−Pi media".

Uptake of [8-^{14}C]adenosine (**2**) was approximately 2 times faster than that of [8-^{14}C]adenine (**1**) in cells in all culture conditions. Cellular concentrations of Pi or ATP (**5**) appear not to influence the velocity of adenosine (**2**) and adenine (**1**) uptake, since the observed velocity in 1-day and 7-day-old *C. roseus* cells was similar. During longer Pi-starvation the velocity of adenosine (**2**) transport gradually decreased, but adenine (**1**) transport was almost constant. Two types of nucleoside transporters, equilibrative and concentrative ones, have been postulated (Li et al., 2003), but only equilibrative nucleoside transporters have been identified in *Arabidopsis thaliana* (Li et al., 2003). One reason for the reduced uptake of adenosine (**2**) and adenine (**1**) in long-term Pi-deficient cells may be the decrease in equilibrative nucleoside transport caused by increasing intracellular levels of adenosine (**2**) and/or adenine (**1**) in the cells.

The metabolic fates of [8-^{14}C]adenosine (**2**) and [8-^{14}C]adenine (**1**) are shown in Figs. 7 and 8. When the distribution of radioactivity is expressed as a percentage of total uptake, incorporation of [8-^{14}C]adenosine (**2**) into nucleotides was kept constant or rather higher in Pi-starved cells than in Pi-fed cells (Fig. 7(a)). Similar trends were found in the experiments using [8-^{14}C]adenine (**1**) (Fig. 8(a)). Adenosine (**2**) and adenine (**1**) are usually salvaged to AMP by adenosine kinase and adenine phosphoribosyltransferase, respectively. AMP is phosphorylated to ADP and finally to ATP (**5**) by adenylate kinase and other enzyme systems, and part of ATP (**5**) is utilised for RNA synthesis. Incorporation of radioactivity from [8-^{14}C]adenosine (**2**) and [8-^{14}C]adenine (**1**) into RNA decreased markedly during long-term Pi starvation (Figs. 7(b) and 8(b)). This seems to reflect the evidence that net RNA synthesis, which is closely related to cell division and growth, does not occur in the Pi-starved cells. Since the energy charges were very low in the Pi-starved cells, it is likely that all biosynthetic pathways, which utilise ATP (**5**), are stopped. Radioactivity from [8-^{14}C]adenosine (**2**) and [8-^{14}C]adenine (**1**) was distributed in ureides (allantoin and allantoic acid) and CO_2 (Figs 7(c) and 8(c)). Since AMP deaminase is a key enzyme for catabolism of adenine nucleotides, and no adenosine (**2**) deaminase is detected in *C. roseus* cells (Yabuki and Ashihara, 1991, 1992), it appears that these degradation products are produced from adenine nucleotides. As shown in a previous paper (Yabuki and Ashihara, 1991), adenine nucleotides are degraded by the conventional catabolic pathway to CO_2 and NH_3 via allantoin and allantoic acid. Approximately 25% of radioactivity from [8-^{14}C]adenosine (**2**) was distributed in degradation

products in the cells up to 14 days, and had almost vanished at day 21. In the case of [8-^{14}C]adenine (**1**), less than 15% of total radioactivity was found in CO_2 and ureides during the entire culture period. The activity of purine catabolism in *C. roseus* cells does not generally increase upon Pi-starvation. Therefore, distinct from the response of other hydrolysing enzymes, enzymes for degradation of purine bases appear not to be induced during Pi-starvation.

The radioactivity from [8-^{14}C]adenosine (**2**) and [8-^{14}C]adenine (**1**) was distributed in adenosine (**2**) and adenine (**1**), respectively (Figs. 8(d) and 9). These compound probably arise from unmetabolised precursors, although we could not exclude the possibility that they were formed from adenine nucleotides that had themselves been formed from these precursors. The proportions of these compounds are greater in the long-term Pi-starved cells. This increase coincides with accumulation of adenosine (**2**) and adenine (**1**) in the cells (Fig. 4).

2.6. Changes in activity of enzymes related to adenosine (2) and adenine (1) salvage during Pi-starvation

Adenosine (**2**) and adenine (**1**) are directly converted to AMP by adenosine kinase and adenine phosphoribosyltransferase, respectively (Moffatt and Ashihara, 2002; Stasolla et al., 2003). In some plants, adenosine (**2**) is hydrolysed to adenine (**1**) by adenosine nucleosidase, and is then salvaged by adenine phosphoribosyltransferase (Koshiishi et al., 2001; Le Floc'h and Faye, 1995). We therefore monitored the activity of these three enzymes (Fig. 9). The activity levels of adenine phosphoribosyltransferase and adenosine (**2**) nucleosidase were higher in cells from "−Pi medium" (days 14–28) than in cells from "+Pi medium" (days 1–7). High adenosine kinase activity was also generally found in Pi-deficient cells, although low activity was found at day 21. Our data suggest that the activity of these purine salvage enzymes is maintained at a high level in the Pi-starved cells.

2.7. Concluding remarks

The transcriptional responses of plants to phosphate starvation are now well documented (Franco-Zorrilla et al., 2004). As a result of the increase in activity of various nucleases, nucleotidases, phosphatases and other hydrolysing enzymes, Pi may be released from cellular organic compounds, such as RNA and nucleotides. However, changes in profiles of phosphorous metabolites during phosphate starvation have not yet been studied in detail. Our results indicate that levels of purine nucleotides, especially ATP (**5**) and GTP, decreased markedly during long-term phosphate starvation, and that AMP and ADP dominated adenine nucleotides, and adenylate energy charge decreased significantly. As a result, the activity of many ATP (**5**)-utilising enzymes may be decreased so that no net biosynthesis of nucleic acids and protein synthesis takes place. Instead, purine nucleosides and bases are accumulated in the cells. Even in Pi-starved cells the activity of purine salvage enzymes is maintained. Recycling of purine nucleoside and bases may therefore sustain the energy required to live under Pi-limited conditions; when reasonable amounts of Pi are supplied, rapid synthesis of nucleotides takes place via the salvage reactions. The proposed turnover mechanism of adenine nucleotides in *C. roseus* cells during long-term Pi starvation is shown schematically in Fig. 10. Studies on nucleotide synthesis in cells during recovery from long-term Pi starvation are in progress.

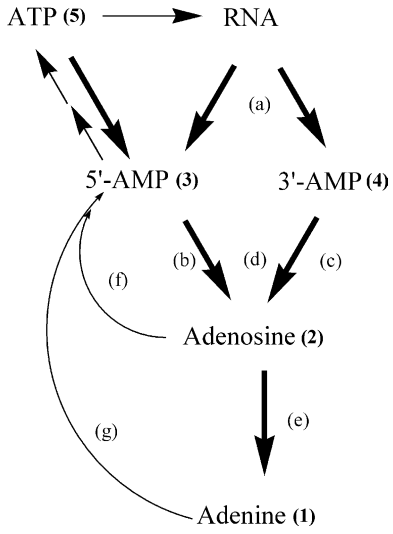

Fig. 10. Turnover of RNA and ATP (**5**) in Pi-starved *Catharanthus roseus* cells. RNA and ATP (**5**) are hydrolysed to adenosine (**2**) releasing Pi. Adenosine (**2**) is further hydrolysed to adenine (**1**). Small amounts of adenosine (**2**) and adenine (**1**) are salvaged to adenine nucleotides. Limited amounts of ATP (**5**) and RNA are maintained during Pi-starvation. A similar turnover pattern is observed with guanine nucleotides. Enzymes: (a) RNase; (b) 5′-nucleotidase; (c) 3′-nucleotidase; (d) acid phosphatase(s); (e) adenosine nucleosidase; (f) adenosine kinase; (g) adenine phosphoribosyltransferase.

3. Experimental

3.1. Chemicals

[8-^{14}C]Adenosine (**2**) (1.85 GBq mmol^{-1}) and [8-^{14}C]-adenine (**1**) (1.96 GBq mmol^{-1}) were obtained from Moravek Biochemicals Inc. (Brea, CA, USA). Standards of nucleotides, nucleosides and nucleobases came from Sigma (St. Louis, MO, USA).

3.2. Plant materials and growth conditions

Stock cultures of *C. roseus* (L.) G. Don were maintained at one-week intervals in the MS basic medium (+Pi medium) containing 1.25 mM Pi, supplemented by 3% sucrose and 2.2 μM 2,4-dichlorophenoxyacetic acid (Murashige and Skoog, 1962). For preparation of experimental cul-

tures, portions (7 ml, ca. 1.5 g fresh weight) of 7-day-old cell suspension were then transferred to 43-ml aliquots of fresh +Pi medium in 300-ml Erlenmeyer flasks for 7 days. The 7-day old cells grown in +Pi medium (7 ml, ca. 1.5 g fresh weight) were transferred to 43-ml Pi-deficient (−Pi) medium and cultured for 3 weeks. The culture flasks were placed on a horizontal rotary shaker (90 strokes min^{-1}, 80 mm amplitude) at 25 °C in the dark.

3.3. HPLC analysis of nucleotides, nucleosides and nucleobases

Nucleotides, nucleosides and nucleobases were extracted from fresh cells (500 mg fresh weight) and were analysed using two HPLC systems, as detailed in Ashihara et al. (1987, 1990) with slight modifications. Nucleotides were analysed using an anion exchange column, Shim-pack WAX-1 (Shimadzu Corporation, Kyoto, Japan) and nucleosides and nucleobases were determined using a polyvinylalcohol gel column, Asahipak GS-320H (Asahi Chemical Industry Co., Kawasaki, Japan). Freshly harvested cells were homogenized in chilled 6% perchloric acid (PCA) with a glass homogeniser. The homogenates were centrifuged at 30,000*g* for 15 min at 2 °C, and the supernatant was collected and neutralized with 20% KOH. After brief centrifuging to remove potassium perchlorate, the samples were lyophilised. The dried samples were dissolved in the solvent for HPLC and were filtered using disposable syringe filter units. Aliquots of 10–50 μl were used for determination by HPLC with a Shimadzu LC 10A HPLC system. The absorbance at 260 nm was monitored using a Shimadzu type SPD-10A, UV–Vis detector. Experiments to assess recovery were performed in parallel with all assays. Known amounts of standard were added to the extraction medium for one member of each pair of duplicate samples prior to homogenisation, and recovery was examined. Recoveries of standards were usually more than 90%. In preliminary experiments, adenylates were also determined by enzymatic analysis, as set out in a previous paper (Kubota and Ashihara, 1993). Essentially the same values were obtained using two different methods. However, some loss of nucleoside tri- and diphosphates was observed when the cells were frozen with liquid nitrogen and stored in a deep-freezer at −80 °C. All assays were therefore performed using freshly harvested cells and were completed on the same day.

3.4. Preparation of enzymes

Freshly harvested *C. roseus* cells (ca. 500 mg fresh weight) were homogenized with 5 ml of 50 mM Tris–HCl buffer (pH 7.5) using a glass homogeniser, and the homogenate was centrifuged at 20,000*g* for 15 min at 2 °C. The resulting supernatant fluid was brought to 80% saturation with finely ground solid ammonium sulphate and was centrifuged at 20,000*g* for 15 min. For determination of the activities of 3′- and 5′-nucleotidases and the acid phosphatase, the ammonium sulphate precipitate was suspended in 50 mM Tris–HCl buffer (pH 7.5), and the sample (2.5 ml) was desalted using a column of Sephadex G-25 (PD-10 column, Amersham Pharmacia Biotech Ltd., UK) that had been equilibrated with the same buffer. The eluted protein fraction (3.5 ml) was used for the assay. For RNase assay, Tris–HCl buffer was replaced by 50 mM succinic acid–NaOH buffer (pH 5.0) containing 2.5 mM sodium EDTA. In the case of adenosine kinase, adenine phosphoribosyltransferase and adenosine nucleosidase, the participating protein was dissolved in 50 mM Hepes–NaOH buffer (pH 7.5) containing 0.5% (w/v) sodium ascorbate and 2 mM 2-mercaptoethanol, and was then desalted.

3.5. Determination of enzyme activity

3.5.1. RNase and DNase

The activity of enzymes was determined by the method of Abel and Glund (1986). The reaction mixture contained 50 mM succinic acid–NaOH buffer (pH 5.0), 250 μg RNA or DNA, and 2.5 mM EDTA; the total volume was 150 μl. The reaction was terminated by adding 1.5 ml of 50 mM acetic acid–NaOH buffer (pH 5.5) containing 50% (v/v) EtOH, 10 mM magnesium acetate and 0.8 mM $La(NO_3)_3$. The mixture was chilled at −20 °C for 45 min and centrifuged at 15,000*g* for 10 min at 4 °C. The absorbance was measured at 260 nm. Enzyme activity was expressed in Wilson units (WU) as defined by Wilson (1975). One WU is the amount of enzyme causing an increase in $A_{1\,cm,\,260\,nm}$ of 1.0 unit min^{-1} in a volume of 1.0 ml.

3.5.2. 5′- and 3′-Nucleotidases and acid phosphatase

Activities were determined by the method of Hirose and Ashihara (1984) with slight modification. The reaction mixture (500 μl) contained 50 mM Tris–HCl buffer (pH 7.5) and (225 μM) 5′-AMP (**3**) or 225 μM 3′-AMP (**4**). The enzyme reactions were terminated by adding (100 μl) of 60% perchloric acid, and the Pi released was determined by the method of Fiske and Subbarow (1925). To estimate 5′- and 3′-nucleotidase, non-specific phosphatase activity at pH 7.5 was measured with (225 μM) glycerophosphate as substrate, and this activity was subtracted from the activities obtained with 5′-AMP (**3**) and 3′-AMP (**4**). The activity of acid phosphatase was measured by the same procedure, but 50 mM succinic acid–NaOH buffer (pH 5.5) and (225 μM) glycerophosphate were used.

3.5.3. Adenine phosphoribosyltransferase, adenosine kinase and adenosine nucleosidase

The activities of these enzymes were determined using labelled substrates as set out in a previous paper (Ashihara et al., 2003). The reaction mixture for adenine phosphoribosyltransferase consisted of 30 mM Hepes–NaOH buffer (pH 7.6), 54 μM [8-^{14}C]adenine (**1**) specific activity, 196 kBq μmol^{-1}, 0.75 mM PRPP, 6.5 mM $MgCl_2$ and 1 mM DTT. The mixture for adenosine kinase contained

30 mM Hepes–NaOH buffer (pH 7.6), 54 μM [8-^{14}C]adenosine (**2**) specific activity, 185 kBq μmol^{-1}, 1.25 mM ATP (**5**), 6.5 mM $MgCl_2$ and 1 mM DTT. The composition of the reaction mixture for adenosine nucleosidase was the same as for adenosine kinase, except that ATP (**5**) was omitted.

3.6. Metabolism of ^{14}C-labelled adenosine (2) and adenine (1)

Adenosine (**2**) and adenine (**1**) were administered into *C. roseus* cells according to the procedure of Ashihara et al. (2000). Suspension-cultured cells (ca. 100 mg fresh weight), and 1.9 ml of the culture medium in which the cells had been grown, were placed in the main compartment of a 30-ml Erlenmeyer flask fitted with a glass tube containing a piece of filter paper impregnated with 0.1 ml of 20% KOH in the centre well. Each reaction was started by adding 10 μl of a solution of labelled compounds to the main compartment of the flask. The flasks were incubated in an oscillating water bath at 27 °C. After incubation, the glass tube was removed from the centre well and placed in a 50-ml Erlenmeyer flask containing 10 ml distilled water. Simultaneously, the cells were harvested by filtration over Miracloth, washed with distilled water, and frozen with liquid nitrogen and temporary stored at −80 °C prior to extraction. Potassium bicarbonate that had been absorbed by the filter paper was allowed to diffuse into distilled water overnight, and aliquots of resulting solution (usually 0.5 ml) were used for the determination of radioactivity. The radioactivity was measured with a liquid scintillation analyser.

The frozen cells were extracted with cold 6% perchloric acid. After extraction, the mixture was centrifuged at 12,000*g* for 7 min and the resulting supernatant was collected. The precipitate was washed with the same extraction reagent, and the supernatant was collected after centrifuging. The perchloric acid-soluble fraction was neutralized with KOH. After removal of the precipitated potassium perchlorate by brief centrifuging, the supernatant was evaporated to dryness in vacuo at 37 °C. The cold perchloric acid-soluble metabolites were then fractionated by TLC using microcrystalline cellulose TLC plates. The solvent systems described by Ashihara and Nobusawa (1981) were used. Radioactivity on the TLC sheet was determined using a Bio-Imaging Analyser (Types FLA-2000, Fuji Photo Film Co., Ltd. Tokyo, Japan).

Nucleic acids (DNA plus RNA) were hydrolysed with 6% perchloric acid at 100 °C for 15 min, and the radioactivity observed in the resulting perchloric acid-soluble metabolites (purine bases derived from nucleic acids) was taken as the radioactivity incorporated into nucleic acids. The radioactivity in liquid fractions was determined using a multi-purpose scintillation counter (type LS 6500, Beckman Instruments, Fullerton, CA, USA).

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