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# Changes in pyridine metabolism profile during growth of trigonelline-forming *Lotus japonicus* cell cultures

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ABSTRACT

Changes in the profile of pyridine metabolism during growth of cells were investigated using trigonelline-forming suspension-cultured cells of *Lotus japonicus*. Activity of the *de novo* and salvage pathways of NAD biosynthesis was estimated from the *in situ* metabolism of [$^3$H] quinolinic acid and [$^{14}$C] nicotinamide. Maximum activity of the *de novo* pathway for NAD synthesis was found in the exponential growth phase, whereas activity of the salvage pathway was increased in the lag phase of cell growth. Expression profiles of some genes related to pyridine metabolism were examined using the expression sequence tags obtained from the *L. japonicus* database. Transcript levels of *NaPRT* and *NIC*, encoding salvage enzymes, were enhanced in the lag phase of cell growth, whereas the maximum expression of *NADS* was found in the exponential growth phase. Correspondingly, the activities of the salvage enzymes, nicotinate phosphoribosyltransferase (EC 2.4.2.11) and nicotinamidase (EC 3.5.1.19), increased one day after transfer of the stationary phase cells to the fresh medium. The greatest *in situ* trigonelline synthesis, both from [$^3$H] quinolinic acid and [$^{14}$C] nicotinamide, was found in the stationary phase of cell growth. The role of trigonelline in leguminous plants is discussed.



## 1. Introduction

Suspension cultured cells comprise a valuable tool for studying growth and differentiation of plant cells. This system has been used to study changes in biosynthesis and utilization of purine and pyrimidine nucleotides during growth of cultured cells (Brown and Short, 1969; Kanamori-Fukuda et al., 1981; Hirose and Ashihara, 1984; Meyer and Wagner, 1985; Wagner and Backer, 1992; Stasolla et al., 2003). In contrast to purine and pyrimidine nucleotides, studies of the biosynthesis and metabolism of pyridine nucleotides and their derivatives in plant cultured cells are limited (Ashihara et al., 2005). Pyridine nucleotides such as NAD (**4**) and NADP (**11**) are produced by the *de novo* pathway via quinolinic acid (**1**), and by the salvage pathway using degradation products of NAD (**4**) (Moat and Foster, 1987; Noctor et al., 2006). Structures of quinolinic acid (**1**) and related compounds are shown in Fig. 1.

Two different *de novo* pathways for pyridine nucleotide biosynthesis have been proposed (Moat and Foster, 1987). In plants, quinolinic acid (**1**) appears to be synthesised from aspartic acid and dihydroxyacetone phosphate by aspartate oxidase and quinolinate synthase (Katoh et al., 2006).

Degradation and regeneration of pyridine nucleotides, i.e., NAD (**4**) recycling, are performed by pyridine nucleotide cycles displaying wide variations (Moat and Foster, 1987; Wagner and Backer, 1992; Ashihara et al., 2005; Noctor et al., 2006). In plants, NAD (**4**) is converted to nicotinamide (**7**) by at least two routes: direct formation from NAD (**4**), releasing ADP-ribose; and a three-step formation via nicotinamide mononucleotide (NMN) (**5**) and nicotinamide riboside (N-riboside) (**6**) (Ashihara et al., 2005; Noctor et al., 2006) (Fig. 2).

Unlike in animals, nicotinamide (**7**) is not used as a substrate for phosphoribosyltransferase (Zheng et al., 2005). Nicotinamide (**7**) is first converted to nicotinic acid (**8**) by nicotinamidase (nicotinamide deaminase, EC 3.5.1.19), and nicotinic acid (**8**) is salvaged to nicotinate mononucleotide (NaMN) (**2**) by nicotinate phosphoribosyltransferase (NaPRT, EC 2.4.2.11). Another route of nicotinic acid (**8**) salvage which involves nicotinate riboside kinase (EC number not yet assigned) may also be operative in plants (Matsui and Ashihara, 2008). Nicotinamide riboside deaminase or nicotinic acid phosphorylase may be involved in this pathway. Nicotinamide riboside deaminase activity has been detected in potato tuber extracts (Katahira and Ashihara, in preparation).

Plants produce various pyridine alkaloids derived from nicotinic acid (**8**). Compared with other pyridine alkaloids, such as ricinine (Yang and Waller, 1965), nicotine and anabasin (Häkkinen et al.,

*Abbreviations:* NaAD, nicotinate adenine nucleotide; NaMN, nicotinate mononucleotide; NaPRT, nicotinate phosphoribosyltransferase; Na-riboside, nicotinate riboside; NMN, nicotinamide mononucleotide; N-riboside, nicotinamide riboside.

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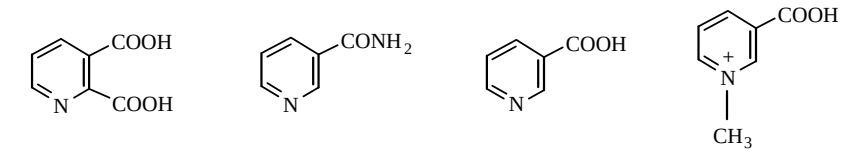


Quinolinic acid (**1**) Nicotinamide (**7**) Nicotinic acid (**8**) Trigonelline (**10**)

**Fig. 1.** Structures of pyridine derivatives.

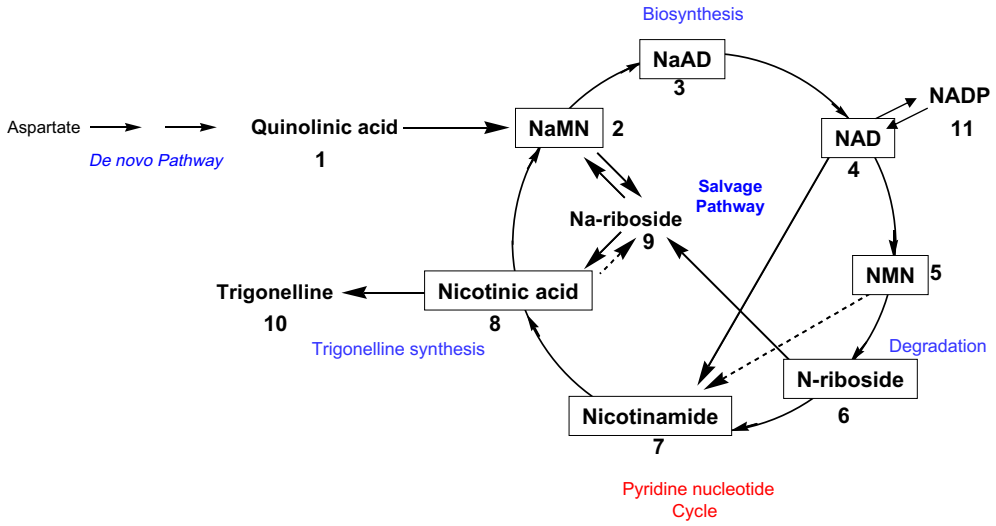


**Fig. 2.** Possible reactions of pyridine metabolism in *Lotus japonicus*. The *de novo* and salvage pathways of pyridine nucleotide biosynthesis, degradation and trigonelline (**10**) synthetic pathway are shown. The recycling cycle of NAD (**4**) is commonly known as the pyridine nucleotide cycle. Reactions shown as solid arrows have been confirmed in plants. Dotted arrows show possible reactions.

2007), trigonelline (**10**) is a more widely distributed pyridine alkaloid, and is found in most leguminous plants (Matsui et al., 2007; Ashihara, 2008). Trigonelline (**10**) is produced from nicotinic acid (**8**) by nicotinate *N*-methyltransferase (EC 2.1.1.7), using *S*-adenosyl-L-methionine as a methyl group donor (Joshi and Handler, 1960; Upmeier et al., 1988; Chen and Wood, 2004), and is important in the symbiosis of leguminous plants and bacteria (Boivin et al., 1990; Phillips et al., 1992).

*Lotus japonicus* is a model organism for leguminous plants and is used for a full genome sequencing project (Pajuelo and Stougaard, 2005); the database contains ESTs for genes which encode enzymes involved in the pyridine cycle (http://www.kazusa.or.jp/lotus/). To incorporate gene expression information in our metabolic studies, we investigated the biosynthesis of pyridine nucleotides and trigonelline (**10**) in cell suspension cultures of *L. japonicus*. We found that the salvage pathway of pyridine nucleotide biosynthesis is activated during the lag phase of cell growth. This activation is due mainly to the increase in nicotinate phosphoribosyltransferase activity as a result of expression of the *NaPRT* gene. Changes in patterns of pyridine metabolism during cell growth and its function are discussed.

## 2. Results and discussion

### 2.1. Growth of *L. japonicus* cultures and changes in NAD (*4*) and trigonelline (*10*) contents

Following inoculation of suspension-cultured starved cells into fresh medium, cells undergo synchronous growth, as well as synchronous uptake and utilisation of the constituents of the medium (Wylegalla et al., 1985; Stasolla et al., 2003). Plant cells cultured under these conditions display very similar growth behaviour. This behaviour can be divided into a lag phase in which no visible change is observed, a cell division phase, characterised by an increase in cell mass, a cell elongation phase caused by increased vacuolation, and a stationary phase once cell growth has ceased (Wagner and Backer, 1992; Stasolla et al., 2003). Fig. 3 shows the growth of our suspension culture of *L. japonicus* together with the changes in the NAD (**4**) and trigonelline (**10**) concentrations during culture.

The fresh weight began to increase two days after the 10-day-old cells were inoculated onto the fresh medium. Exponential growth commenced at day 4, and continued to day 7. After day 10, the growth reached the stationary phase. The length of the lag phase appears to depend on the duration of the preceding starvation phase. Although we monitored growth until day 14 (Fig. 3a), some cells deteriorated after 10 days. We therefore chose a 10 day growth cycle in this study. The pattern of change in growth was similar to that reported in *Catharanthus roseus* cultures (Kanamori et al., 1979). Similar patterns of growth have also been observed in suspension cultures of other species, such as sycamore (Brown and Short, 1969) and *Datura innoxia* (Meyer and Wagner, 1985). We performed experiments on cells from the cultures at the initial phase (day 0), the lag phase (day 1), the cell division phase (day 4), the cell expansion phase (day 7) and the stationary phase (day 10).

The NAD (**4**) concentration increased markedly in the lag phase, and the maximum value (nearly 3 times the initial value) was observed at day 4. After that the concentrations fell substantially (Fig. 3b). Similar changes in NAD (**4**) concentration have been found during growth of batch suspension cultures of *Datura innoxia*; NAD (**4**) concentration increased to more than 5 times

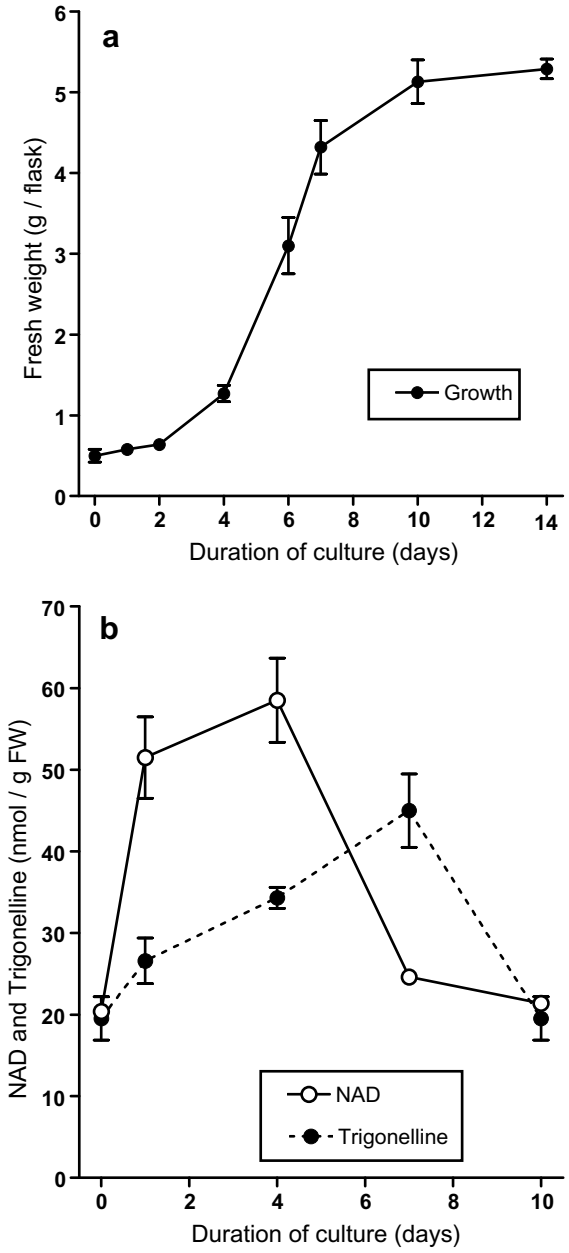


**Fig. 3.** Changes in growth (a) and NAD (**4**) and trigonelline (**10**) concentrations (b) during growth of suspension-cultured *Lotus japonicus* cells. Growth is shown as the mean and SD of g fresh weight per flask. Mean concentrations of metabolites are expressed as nmol per fresh weight with SD.

the initial value in the early phase (day 4), then decreased (Meyer and Wagner, 1985).

In contrast, trigonelline (**10**) concentration increased gradually from 20 to 45 nmol/g fresh weight up to day 7 of culture, then returning to approximately 20 nmol/g by day 10 (Fig. 3b). Some of the trigonelline (**10**) generated in cultured cells was released to the culture medium in the later stages of culture; considerable amounts of trigonelline (**10**) (485 ± 33 nmol/flask, equivalent to 9.7 ± 0.7 nmol/ml) were detected in medium collected from the 10-day-old culture. Trigonelline (**10**) content in seeds has been reported (Tramontano et al., 1986; Matsui et al., 2007), but there is little information on the endogenous concentration of trigonelline (**10**) in growing cells. The concentration observed in cultured *L. japonicus* cells is much lower than in seeds (0.9 μmol per g fresh weight) of this plant (Matsui et al., 2007).

### 2.2. Metabolism of $^{3}$H-quinolinic acid (**1**) and $^{14}$C-nicotinamide (**7**) in *L. japonicus* cells

Quinolinic acid (**1**) is an intermediate of *de novo* pyridine nucleotide synthesis, whereas nicotinamide (**7**) is a catabolite of NAD (**4**) and can be used for regeneration of NAD (**4**) by the salvage pathway (Fig. 2). These two compounds were labelled with $^{3}$H or $^{14}$C and were used for estimation of the *in situ* activity of the *de novo* and salvage pathways.

The metabolism of [$^{3}$H] quinolinic acid (**1**) and [carbonyl-$^{14}$C] nicotinamide (**7**) in *L. japonicus* cells was examined using two different incubation time, of 4 and 18 h. Fig. 4 shows typical results from these experiments. [$^{3}$H] Quinolinic acid (**1**) taken up by the cells was metabolised readily, and radioactivity was found only in NAD (**4**), NADP (**11**), trigonelline (**10**), NaMN (**2**), NMN (**5**) and unmetabolised quinolinic acid (**1**). When the incorporation of radioactivity into each compound is expressed as % of total uptake, the distribution of $^{3}$H in each metabolite at 4 h- and 18 h-incubation was similar (Fig. 4a).

[Carbonyl-$^{14}$C] nicotinamide (**7**) taken up by the cells appeared to be immediately converted to nicotinic acid (**8**) and then metabolised, since no radioactivity was detected in nicotinamide (**7**) even after a shorter incubation. A substantial amount of radioactivity was found in pyridine nucleotides, especially NAD (**4**) and NADP (**11**). More than 20% of radioactivity was found in nicotinic acid (**8**) at 4 h after incubation. Radioactivity in nicotinic acid (**8**) decreased during the longer incubation time, but rates of incorporation into nucleotides and trigonelline (**10**) increased slightly (Fig. 4b).

Our results suggest that almost all [$^{3}$H] quinolinic acid (**1**) taken up by *L. japonicus* cells is converted to NaMN (**2**) and utilised for pyridine nucleotide and trigonelline (**10**) synthesis, according to the pathways shown in Fig. 2. Since significant incorporation of radioactivity from [$^{3}$H] quinolinic acid (**1**) into trigonelline (**10**) was found even in the shorter incubation period of 4 h, a considerable amount of trigonelline (**10**) is formed directly from quinolinic acid (**1**) via NaMN (**2**), but not via the pyridine cycle. Direct formation of trigonelline (**10**) has been proposed in mungbean seedlings (Zheng et al., 2008). Conversion of [$^{14}$C] nicotinamide (**7**) to [$^{14}$C] nicotinic acid (**8**) was catalysed probably by nicotinamidase in *L. japonicus* cells, because no radioactivity was detected in nicotinamide (**7**) and a high activity of nicotinamidase was found in all stages of cultured cells (Fig. 8). Rapid deamination of nicotinamide (**7**) supplied exogenously to plant tissues has also been observed in leaves of *Arabidopsis thaliana* (Wang and Pichersky, 2007) and in developing *Brassica napus* embryos (Ashihara et al., 2008).

Pyridine nucleotides appear to be synthesised from nicotinamide (**7**) via nicotinic acid (**8**) by the routes shown in Fig. 2. As in other plants (Zheng et al., 2004, 2005; Matsui et al., 2007), the major pathway of nicotinamide (**7**) salvage in *L. japonicus* is a nicotinamide (**7**) → nicotinic acid (**8**) → NaMN (**2**) → NaAD (**3**) → NAD (**4**) pathway. Trigonelline (**10**) is synthesised via nicotinic acid (**8**). Little or no degradation of nicotinic acid (**8**) appears to have occurred during 18 h, because no radioactivity was found in possible degradation products, including $CO_2$ (Barz, 1985).

### 2.3. Changes in metabolic profile of quinolinic acid (**1**) and nicotinamide (**7**) during growth

To examine the contribution of *de novo* and salvage pathways to nucleotide and trigonelline (**10**) synthesis during growth of *L. japonicus* cells, we monitored the metabolic fate of [$^{3}$H] quinolinic acid (**1**) and [$^{14}$C] nicotinamide (**7**) 18 h after the labelled precursors were administered to the cells in different growth stages.

Fig. 5 shows the uptake of exogenously administered [$^{3}$H] quinolinic acid (**1**) and [$^{14}$C] nicotinamide (**7**) by *L. japonicus* cells. The

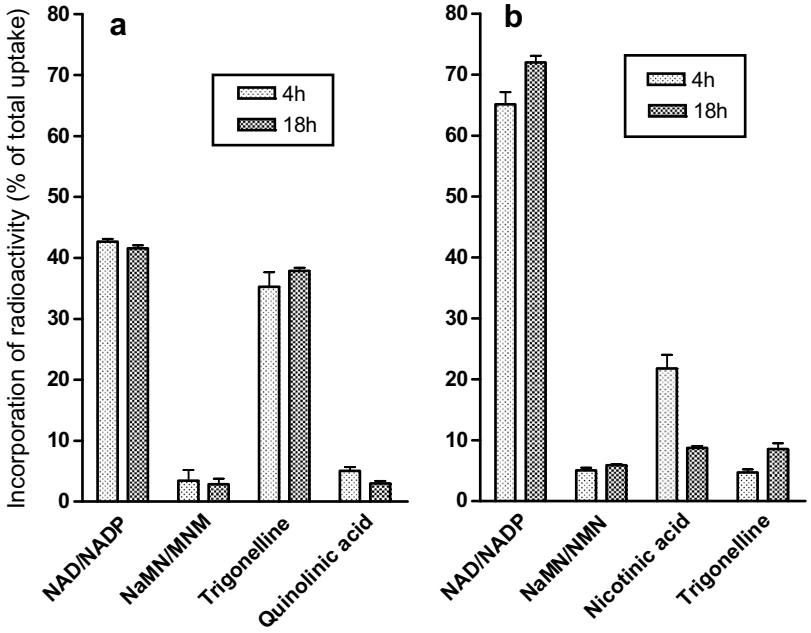


**Fig. 4.** Metabolism of [$^3$H] quinolinic acid (**1**) (a) and [$^{14}$C] nicotinamide (**7**) (b) in suspension-cultured *Lotus japonicus* cells. Incubation with labelled compounds took place for 4 h and 18 h. Incorporation of radioactivity is expressed as % of total uptake. Mean values and SD are shown. The results shown are from 7-day-old (a) and 1-day-old (b) cells.

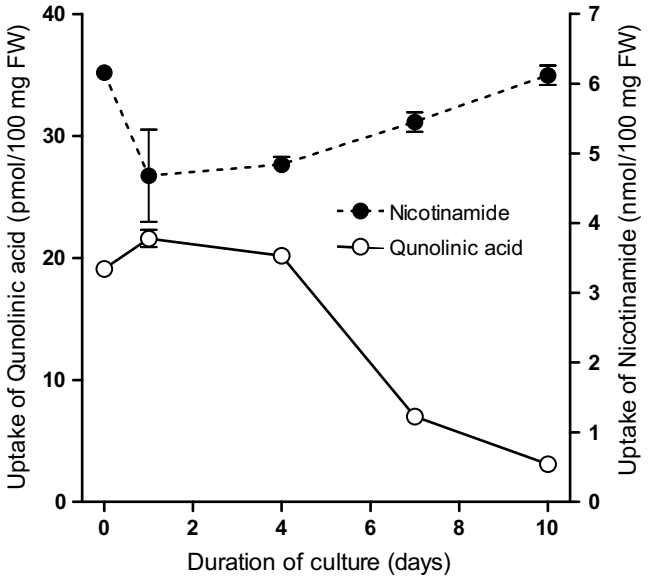


**Fig. 5.** Uptake of [$^3$H] quinolinic acid (**1**) (a) and [$^{14}$C] nicotinamide (**7**) (b) in suspension-cultured *Lotus japonicus* cells in different growth phases. Incubation with labelled compounds took place for 18 h. The uptake of labelled compounds is expressed as nmol per 100 mg fresh weight cells. Mean values and SD are shown.

rate of uptake of [$^3$H] quinolinic acid (**1**) was much lower than that of [$^{14}$C] nicotinamide (**7**). Similar observations have been made in animal tissues. Ijichi et al. (1966) reported that [$^{14}$C] nicotinamide (**7**) penetrated more readily into liver cells than [$^{14}$C] quinolinic acid (**1**). The rate of uptake of [$^3$H] quinolinic acid (**1**) by *L. japonicus* cells was dependent on the age of the cell culture; uptake rates decreased markedly in the later stages. Changes in the components of the medium (nutrients and/or ions) may induce differing uptakes of quinolinic acid (**1**). When 10-day-old cells were transferred to the fresh medium, uptake of [$^3$H] quinolinic acid (**1**) increased dramatically (Fig. 5). In contrast, the rate of nicotinamide (**7**) uptake is always high, especially in the later stages of cell growth. Nicotinamide (**7**), which is a substrate of the pyridine nucleotide salvage, may be absorbed by transport systems, although the specific transporter has not yet been isolated (Reyes et al., 2002). In contrast, penetration of quinolinic acid (**1**), which is an intermediate of the *de novo* pyridine nucleotide synthesis, does not occur in nature if it is not applied artificially.

A simplified analysis suffices for comparison of metabolic fate, so that we group the radiolabelled metabolites into three classes as shown in Fig. 6. The radioactivity distributed in the pyridine nucleotides consisted of NAD (**4**), NADP (**11**), NaMN (**2**) and NMN (**5**) (Fig. 6a and d), trigonelline (**10**) (Fig. 6b and e), and unmetabolised quinolinic acid (**1**) and nicotinic acid (**8**), respectively. Fig. 6c and f, expressed as a percentage of total uptake.

Incorporation of radioactivity from [$^3$H] quinolinic acid (**1**) to pyridine nucleotides increased gradually after the cells were transferred to the fresh medium. The maximum (comprising 65% of total radioactivity) was found at day 4, after which it decreased (Fig. 6a). In contrast, the maximum rate of conversion of [$^{14}$C]nicotinamide (**7**) to pyridine nucleotides (71% of total radioactivity) was found in the cells of the lag phase (day 1), and the rate was almost constant (at 37–44%) after day 4 (Fig. 6d). Incorporation of radioactivity into trigonelline (**10**) from both [$^3$H] quinolinic acid (**1**) and from [$^{14}$C] nicotinamide (**7**) decreased after cells were transferred to the fresh medium, then increased (Fig. 6b and e).

The patterns of trigonelline (**10**) synthesis and nucleotide biosynthesis from [$^3$H] quinolinic acid (**1**) are mirror images of each other. A similar profile was found in the experiments with [$^{14}$C] nicotinamide (**7**), but no mirror image was found during the later stage of culture (days 4–10). Little unmetabolised quinolinic acid (**1**) was detected. In contrast, approximately 20% of radioactivity was found in nicotinic acid (**8**), except in 1-day-old cells when [$^{14}$C] nicotinamide (**7**) was supplied.

These results suggest that the contribution of the *de novo* pathway for pyridine biosynthesis is significant in the exponential growth phase beginning with cell division. In contrast, the salvage pathway of nicotinamide (**7**) is key in the lag phase of cell growth where any substantial growth of cells takes place. Except for this

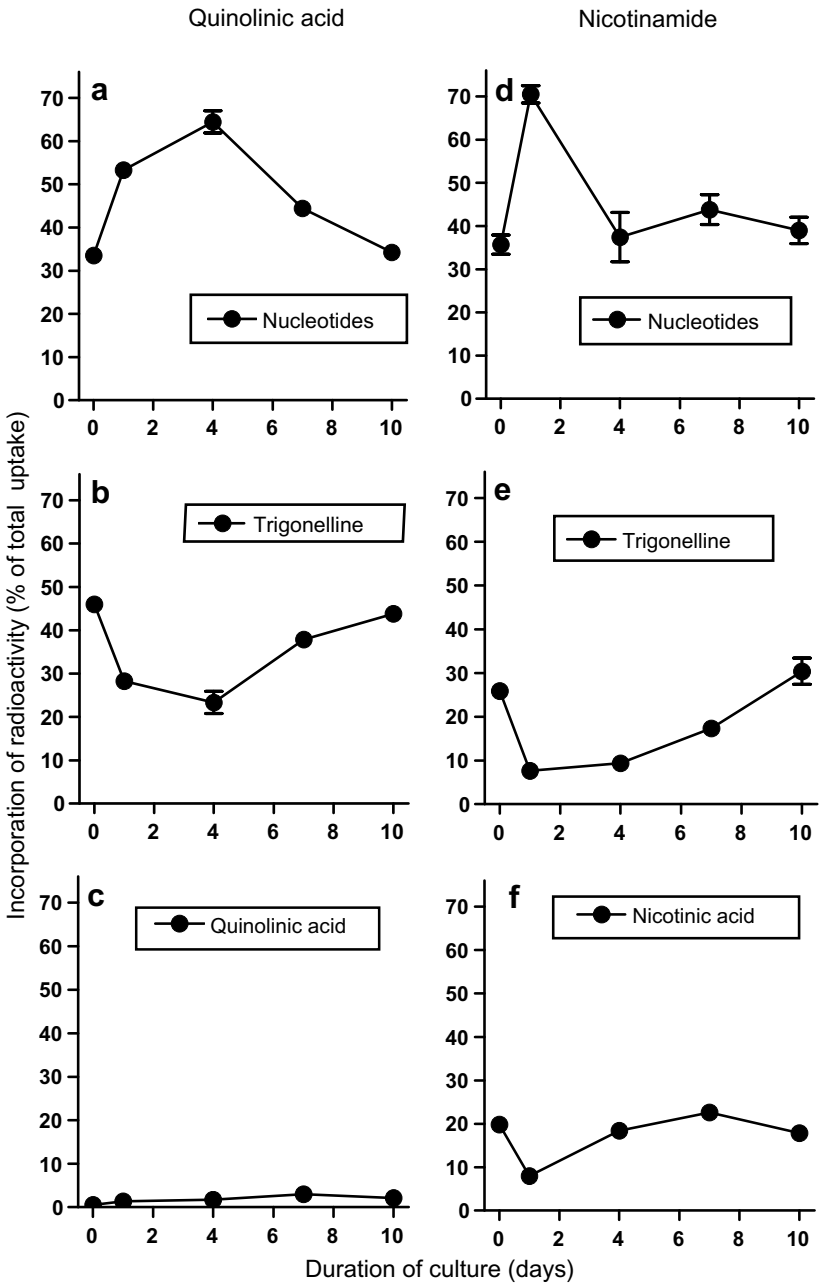


**Fig. 6.** Metabolic fate of [$^{3}$H] quinolinic acid (**1**) (a–c) and [$^{14}$C] nicotinamide (**7**) (d–f) in suspension-cultured *Lotus japonicus* cells of different growth phases. Incubation with labelled compounds took place for 18 h. The distribution of radioactivity in pyridine nucleotides (a and d), trigonelline (**10**) (b and e), quinolinic acid (**1**) (c) and nicotinic acid (**8**) (f) is expressed as % of total activity taken up by the cells, as shown in Fig. 5. Mean values and SD are shown.

stage, the salvage activity is almost constant during cell growth. Trigonelline (**10**) synthesis from both quinolinic acid (**1**) and nicotinamide (**7**) is higher in the stationary phase cells than in actively growing cells.

### 2.4. Changes in expression of genes encoding the pyridine nucleotide cycle

The *L. japonicus* EST database records genes encoding some enzymes of the pyridine nucleotide cycle. Using the sequences for NaPRT (*NaPRT*), nicotinamidase (*NIC*), NAD synthetase (*NADS*) and actin (*Actin*) as constitutive controls, we examined changes in the transcript levels of these genes in *L. japonicus* cells during culture, as shown in Fig. 7. The results indicated that transcript levels change during growth of the cell cultures. The levels of *NaPRT* and *NIC* transcripts were low at day 0, increased markedly at day 1, then decreased slightly. In contrast, the transcript level of *NADS* increased gradually up to the cell division phase (day 4), then fell. The amount of transcripts of *Actin* in any growth stage of the culture scarcely varied.

### 2.5. Changes in activity of NaPRT and nicotinamidase

*In situ* tracer- and gene expression-experiments suggest that salvage of nicotinamide (**7**) for pyridine nucleotide synthesis increases in the lag phase of cell growth. To determine whether nicotinamide (**7**) salvage is caused by an increased activity of NaPRT (EC 2.4.2.11) and/or nicotinamidase (EC 3.5.1.19), we monitored the *in vitro* enzyme activity of these two enzymes using an enzyme preparation obtained from various stages of the cultures (Fig. 8).

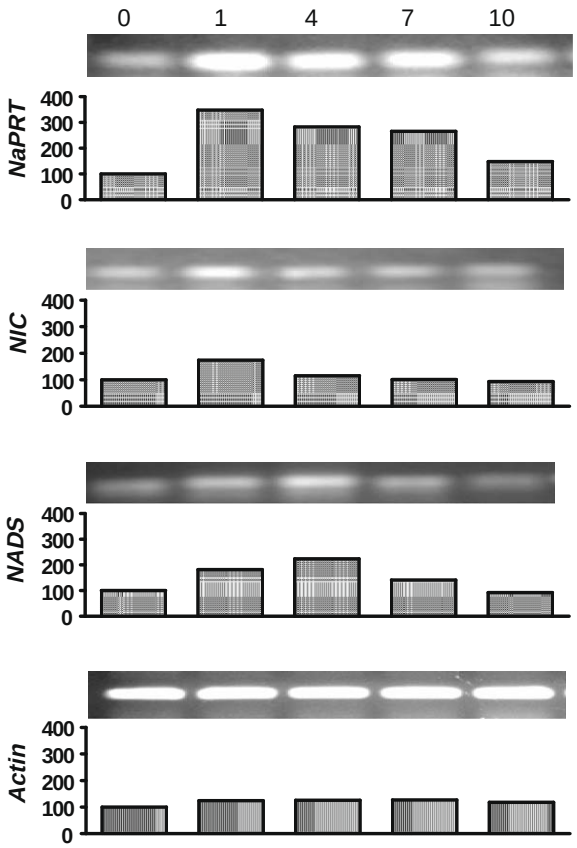


**Fig. 7.** Expression of *NaPRT*, *NIC*, *NADS* and *Actin* in suspension-cultured *Lotus japonicus* cells. Relative amounts of transcripts are shown as % of values from 0-day-old cells for the different growth phases. Results obtained by 26 cycles (*NIC*) or 30 cycles (*NaPRT* and *Actin*) of PCR amplification are shown. The bar graphs show relative intensities, expressed as % relative to the sample at the start (day 0).

Activity of NaPRT increased almost fivefold one day after the stationary phase cells were transferred to the fresh medium. The activity of NaPRT decreased gradually with cell growth (Fig. 8a). Nicotinamidase activity almost doubled on day 1 (Fig. 8b).

*In vitro* activity of nicotinamidase was much higher than NaPRT activity at all stages of cell growth; even at day 1, nicotinamidase activity was 25 times higher than NaPRT activity. Furthermore, *in situ* experiments with [$^{14}$C] nicotinamide (**7**) shows that nicotinamide (**7**) was readily converted to nicotinic acid (**8**) in *L. japonicus* cells. The rate limiting step in nicotinamide (**7**) salvage is therefore the activity of NaPRT.

### 2.6. Concluding remarks

Our studies suggest that the relative contribution of *de novo* and salvage pathways to pyridine nucleotide synthesis depends on the growth stages of *L. japonicus* cells. Acceleration of nicotinamide (**7**) salvage in the lag phase of cell growth is accomplished by the expression of *NaPRT* and *NIC*, and by increased activity of NaPRT and nicotinamidase. A marked increase in the activity of these two enzymes was also reported in *Catharanthus roseus* cells when the cells recovered from phosphate starvation (Yin et al., 2007). Since reduced forms of NAD (**4**) and NADP (**11**) are closely related to the production of energy in mitochondria and the supply of reducing power for nitrate reduction and synthesis of cellular constituents required for the initiation of cell division, pyridine salvage is important in generating increased levels of net NAD (**4**) and NADP (**11**). Salvage pathways in the lag stage of cell growth are known to be important in purine and pyrimidine nucleotide biosynthesis (Kanamori-Fukuda et al., 1981; Shimazaki and Ashihara, 1982).

The increase in *de novo* synthesis of pyridine nucleotides was accompanied by exponential growth of the cells. The biphasic changing pattern of nucleotide biosynthesis, such that the peak of the salvage pathway precedes the *de novo* pathways, has also been found in pyrimidine biosynthesis in *Catharanthus roseus* cells (Kanamori-Fukuda et al., 1981). These results suggest that, in the lag phase, the cells use small amounts of pyridine compounds, as well as purine bases and nucleosides present in the stationary phase cells, for pyridine nucleotide synthesis by salvage enzymes. The *de novo* synthesis of pyridine nucleotides observed in the exponential phase is presumably triggered by the increased recycling of these nucleotides by the salvage pathways. Although we did not detect nicotinic acid (**8**) in cells in the stationary phase, there was accumulation of purine nucleosides and bases in phosphate deficient culture medium (Shimano and Ashihara, 2006).

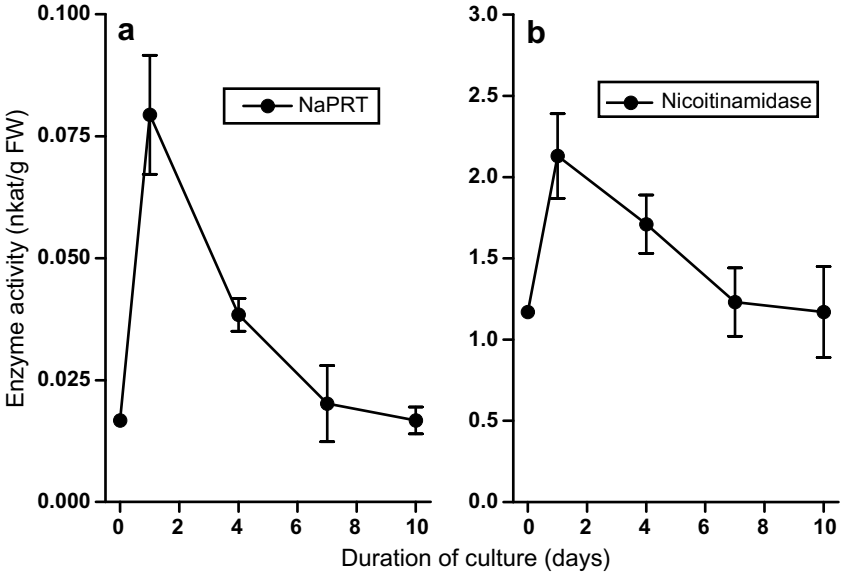


**Fig. 8.** Changes in activities of nicotinate phosphoribosyltransferase (NaPRT) (a) and nicotinamidase (b) in suspension-cultured *Lotus japonicus* cells during culture. Enzyme activities are expressed as nkat per g fresh weight cells.

Our present study suggests that trigonelline (**10**) is produced mainly when pyridine nucleotide synthesis decreases. A significant portion of trigonelline (**10**) formed in the cells is released to the culture medium. It is possible that trigonelline (**10**) acts as a reservoir of nicotinic acid (**8**), because trigonelline demethylase activity is found in plants (Taguchi and Shimabayashi, 1983; Shimizu and Mazzafera, 2000). Trigonelline (**10**) catabolism may be more important in nodules of leguminous plants. It has been reported that trigonelline (**10**) is excreted from seeds of *Medicago sativa*, and induces nodulation (*Nod*) gene transcription in *Rhizobium meliloti* by activating the regulatory protein NodD2 (Phillips et al., 1992); *R. meliloti* genes encoding catabolism of trigonelline (**10**) are induced under symbiotic conditions (Boivin et al., 1990). Trigonelline (**10**) may therefore be intimately related to the symbiosis of *L. japonicus* and leguminous bacteria, *Mesorhizobium loti* and/or *Bradyrhizobium* sp. (*Lotus*) strains (Pajuelo and Stougaard, 2005). In *L. japonicus* plants, nicotinic acid (**8**) salvage activity was much higher in nodules than in other parts (Matsui et al., 2007). Trigonelline (**10**) produced in leguminous plants may be transported to root nodules and utilised for NAD (**4**) cycling.

## 3. Experimental

### 3.1. Plant materials

*Lotus japonicus* seeds were obtained from the National Bioresource Project (*L. japonicus* and *G. max*) Core Facility Office, Department of Agriculture, Miyazaki University, Miyazaki, Japan. A suspension culture of *L. japonicus* Gifu B-129 was established from the callus initiated from hypocotyls of dark-grown seedlings, using Murashige–Skoog basic salt medium (Murashige and Skoog, 1962) supplemented by 3% sucrose, 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μM kinetin. Stock suspension cultures were maintained at 10 day intervals in Murashige–Skoog basic salt medium supplemented by 0.3 μM thiamine, 560 μM myo-inositol, 27 μM glycine, 3% sucrose and 4.5 μM 2,4-D.

For preparation of experimental cultures, portions of 10-day-old cell suspension (7 ml, ca. 500 mg fresh weight) were transferred to 43-ml aliquots of fresh medium in 300-ml Erlenmeyer flasks, and were cultured for 10 days. The culture flasks were placed on a horizontal rotary shaker (90 strokes $min^{-1}$, 80 mm amplitude) at 25 °C in the dark.

### 3.2. Radiochemicals and biochemicals

[$^3$H] Quinolinic acid (**1**) (specific activity 167 GBq $mmol^{-1}$) and [carbonyl-$^{14}$C] nicotinamide (**7**) (specific activity 2.04 GBq $mmol^{-1}$) were obtained from Moravek Biochemicals, Inc., Brea, CA, USA. Biochemicals were purchased from Sigma–Aldrich, St. Louis, Mo, USA.

### 3.3. Determination of NAD (*4*) and trigonelline (*10*)

NAD (**4**) and trigonelline (**10**) was analysed by the method of Sestini et al. (2000) and Zheng and Ashihara (2004). NAD (**4**) was extracted with 1 M $HClO_4$ and neutralized with 3.6 M KOH, and $KHClO_4$ was removed by centrifuging. The resultant supernatant was lyophilized. Samples were dissolved in 100 mM $KH_2PO_4$ containing 6 mM TBA (pH 5.6) and were used for HPLC analysis. Trigonelline (**10**) was extracted with $H_2O$–MeOH (1:4, v/v) followed by vacuum drying. After complete evaporation of the MeOH, the MeOH-soluble extracts were dissolved in distilled $H_2O$. HPLC was carried out with a Shimadzu LC 10A HPLC system and a Shimadzu Diode Array Detector, type SPD-M10A (Shimadzu Corporation, Kyoto, Japan). Columns used for NAD (**4**) and trigonelline (**10**) analysis were, respectively, an Ultrasphere ODS column (3 μm particle size, 75 × 4.6 mm, Beckman Coulter, Inc., Fullerton CA, USA) and an ODS Hypersil column (5 μm particle size, 250 × 4.6 mm, Shandon, Runcorn, Cheshire, UK). Elution programs are the same as in the original protocols (Sestini et al., 2000; Zheng and Ashihara, 2004).

### 3.4. Tracer experiments using intact cells

Suspension-cultured cells (ca. 100 mg fresh weight) and culture medium (2 ml) 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 3.6 M KOH in a centre well, to collect $^{14}CO_2$. For [$^3$H] quinolinic acid (**1**), flasks with no centre well were used. Each reaction was started by adding 10 μl of a solution of [$^3$H] quinolinic acid (**1**) (370 kBq, specific activity 167 GBq $mmol^{-1}$) or [carbonyl-$^{14}$C] nicotinamide (**7**) (37 kBq, specific activity 2.04 GBq $mmol^{-1}$) 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 distilled $H_2O$ (10 ml). At the same time, the cells were harvested by filtration over Miracloth, washed with distilled $H_2O$, and frozen with liquid $N_2$. $KHCO_3$ that had been absorbed by the filter paper was allowed to diffuse into distilled $H_2O$ overnight, and aliquots of the resulting solution (usually 0.5 ml) were used for determination of radioactivity. Radioactivity was measured with a liquid scintillation analyzer.

The frozen cells were extracted with cold $H_2O$–MeOH (1:4, v/v) 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 in $H_2O$–MeOH (1:4, v/v), and supernatant was collected by centrifugation. The first and second extracts from MeOH were combined and evaporated *in vacuo* to dryness. The MeOH-soluble metabolites were then fractionated by TLC using microcrystalline cellulose TLC plates (Merck, Darmstadt, Germany). Solvent systems used were (I) *n*-BuOH–HOAc–$H_2O$ (4:1:2, v/v/v) and (II) isobutyric acid–$NH_3$–$H_2O$ (660:17:330, v/v/v) (Matsui et al., 2007).

Radioactivity of $^{14}$C-metabolites on the TLC sheet was determined using a Bio-Imaging Analyzer (FLA-2000, Fuji Photo Film Co. Ltd., Tokyo, Japan). Spots of $^3$H on the TLC were scraped off and $^3$H-metabolites were eluted from the cellulose support with $H_2O$–MeOH (1:1, v/v). Radioactivity was determined using a liquid scintillation counter (Beckman, type LS 6500).

### 3.5. Semi-quantitative RT-PCR

Total RNA was extracted from *L. japonicus* cells at various stages of growth, and was purified using the method reported previously (Li et al., 2008; Deng et al., 2008). DNA-free total RNA was employed for first strand cDNA synthesis, using a RNA PCR core kit (Applied Biosystems, Roche Molecular systems, USA). The PCR reaction mixture (25 μl) contained 40 ng cDNA and 12.5 μl GoTaq Green mastermix (Invitrogen, Carlsbad, CA, USA); 25–30 cycles were carried out using a program of 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min. The reaction product was visualized by UV light on 2% agarose gels stained with ethidium bromide, using a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan). The amounts of products resulting were determined densitometrically after gel electrophoresis, to find the relative level of transcripts of each gene. Gene specific primers were prepared from the sequences registered in the Genbank. The sequences of primers used in this work were as follows: *NaPRT* (BP041001): 5′-TTTGGTGCACTCAATGGGTA-3′, 5′-TGGATGTGTTGAGAGCAAGC-3′; *NIC* (BP044436): 5′-GAAGAAATCCCGGTGAAACA-3′, 5′-AGGCCAATTCTTCTCAGCAA-3′; *NADS* (BP048724): 5′-GCCTCTGCCTGTCAAAGAAC-3′, 5′-GATCCATCTTTCCCCCTTTC-3′;

*Actin* (AU089544): 5′-CTTTTAATACCCCCGCTATGTATG-3′, 5′-GGTGGTAAAAGAATAACCACGTTC-3′.

### 3.6. Determination of enzyme activity

Freshly harvested *L. japonicus* cells (ca. 500 mg fresh weight) were homogenized in the extraction medium, consisting of 50 mM HEPES-NaOH buffer (pH 7.6), 2 mM sodium EDTA, 0.14% 2-mercapto ethanol and 2% PVP, using a glass homogeniser on ice; the homogenate was centrifuged at 30,000*g* for 20 min at 2 °C. The supernatant was treated with 80% saturated $(NH_4)_2SO_4$, and the precipitate was collected by centrifuging at 30,000*g* for 10 min. The pellet was dissolved in 50 mM HEPES-NaOH buffer (pH 7.6), and was desalted on a pre-packed Sephadex G-25 (PD-10) column (GE Healthcare, Tokyo, Japan) that had been equilibrated with 25 mM HEPES-NaOH buffer (pH 7.6).

Enzyme activities were determined using $^{14}C$-labelled substrates. The total volume of reaction mixtures was 100 μl. Reaction mixtures for enzyme assay contained the following components:

NaPRT: 50 mM HEPES-NaOH buffer (pH 7.6), 20 μM [carboxyl-$^{14}C$] nicotinic acid (**8**) (3.7 kBq, specific activity 1.85 GBq mmol$^{-1}$), 0.75 mM PRPP, 1 mM ATP and 10 mM $MgCl_2$.

Nicotinamidase: 50 mM HEPES-NaOH buffer (pH 7.6), 17 μM [carbonyl-$^{14}C$] nicotinamide (**7**) (3.7 kBq, specific activity 2.04 GBq mmol$^{-1}$).

The reaction mixture was incubated at 27 °C. Enzyme reactions were terminated by transferring the test tubes to a boiling water bath for 2 min. Denatured protein was removed by centrifuging at 12,000*g* for 5 min, and the supernatant was evaporated *in vacuo* to dryness. The dried pellet was dissolved in small amounts (ca. 200 μl) of $H_2O$–EtOH (1:1, v/v) and then centrifuged at 12,000*g* for 5 min. An aliquot of supernatant (ca. 10 μl) was loaded onto the cellulose TLC plate. Labelled substrate and product were separated by TLC using the solvent system I (NaPRT) or II (nicotinamidase) shown in Section 3.4. The reaction of NaPRT was linear for at least 30 min, and that of nicotinamidase for 10 min.

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