

Metabolic fate of nicotinamide in higher plants

Ayu Matsui^a, Yuling Yin^a, Keiko Yamanaka^b, Midori Iwasaki^b and Hiroshi Ashihara^{a,b,*}

^aDepartment of Biological Sciences, Graduate School of Humanities and Sciences, Ochanomizu University, Bunkyo-ku, Tokyo 112-8610, Japan

^bDepartment of Biology, Faculty of Science, Ochanomizu University, Bunkyo-ku, Tokyo 112-8610, Japan

Correspondence

*Corresponding author,

e-mail: ashihara.hiroshi@ocha.ac.jp

Received 6 May 2007; revised 9 June 2007

doi: 10.1111/j.1399-3054.2007.00959.x

Metabolism of [carbonyl-¹⁴C]nicotinamide was surveyed in various plant materials including the model plants, *Arabidopsis thaliana*, *Oryza sativa* and *Lotus japonicus*. In all plants studied, nicotinamide was used for the pyridine (nicotinamide adenine) nucleotide synthesis, probably after conversion to nicotinic acid. Radioactivity from [carbonyl-¹⁴C]nicotinamide was incorporated into trigonelline (1-*N*-methylnicotinic acid) and/or into nicotinic acid 1*N*-glucoside (Na-Glc). Trigonelline is formed mainly in leaves and cell cultures of *O. sativa* and *L. japonicus* and in seedlings of *Trifolium incarnatum*, *Medicago sativa* and *Raphanus sativus*. Trigonelline synthesis from nicotinamide is generally greater in leaves than in roots. Na-Glc was formed as the major nicotinic acid conjugate in *A. thaliana* and in tobacco Bright Yellow-2 cells. In seedlings of *Chrysanthemum coronarium* and *Theobroma cacao*, both trigonelline and Na-Glc were synthesized from [carbonyl-¹⁴C]nicotinamide. Trigonelline is accumulated in some seeds, mainly Leguminosae species. The pattern of formation of the nicotinic acid conjugates differs between species and organs.

Introduction

Nicotinamide is formed as a catabolite of nicotinamide adenine nucleotide and is a key metabolite of pyridine metabolism. It is similar to purine and pyrimidine bases (Ashihara and Crozier 1999, Moffatt and Ashihara 2002, Stasolla et al. 2003, Zrenner et al. 2006); this pyridine base is salvaged to nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN) and is used for regeneration of NAD and NADP (Ashihara et al. 2005). Less is known about pyridine metabolism in plants (Kato and Hashimoto 2004, Noctor et al. 2006) than in mammals or microorganisms (Moat and Foster 1987). Some differences between plants and animals are known. In mammals, nicotinamide is salvaged to NMN directly by nicotinamide phosphoribosyltransferase (EC 2.4.2.12) or by two-step reactions via nicotinamide riboside (NR) (Magni et al. 1999). Although nicotinamide is not deaminated to nicotinic acid in mammals, exogenously supplied nicotinic

acid as diet is salvaged by nicotinate phosphoribosyltransferase (EC 2.4.2.11) to NaMN and is used in NAD synthesis (Brenner 2005, Revollo et al. 2004, Sestini et al. 2000). Plants lack nicotinamide phosphoribosyltransferase, so that the pathway via NaMN is functional; nicotinamide is converted to nicotinic acid, which is then phosphoribosylated in plants (Ashihara et al. 2005, Zheng et al. 2005). Outlines of the metabolic pathways are shown in Fig. 1.

In addition to salvage, nicotinamide and nicotinic acid are used in a few plants for the synthesis of alkaloids such as nicotine (Kato and Hashimoto 2004) and ricinine (Waller et al. 1966), in very limited plants, such as tobacco and castor bean plants for instance. A simple and ubiquitous alkaloid is derived from nicotinic acid in the one-step conversion to trigonelline (1-*N*-methylnicotinic acid) by an *N*-methyltransferase. In the 1980s, Tramontano et al. (1983) and Barz (1985) published reports on trigonelline formation and its possible functions,

Abbreviations – NaAD, nicotinic acid adenine dinucleotide; Na-Glc, nicotinic acid 1*N*-glucoside; NaMN, nicotinic acid mononucleotide; NaR, nicotinic acid riboside; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; PNC, pyridine nucleotide cycle.

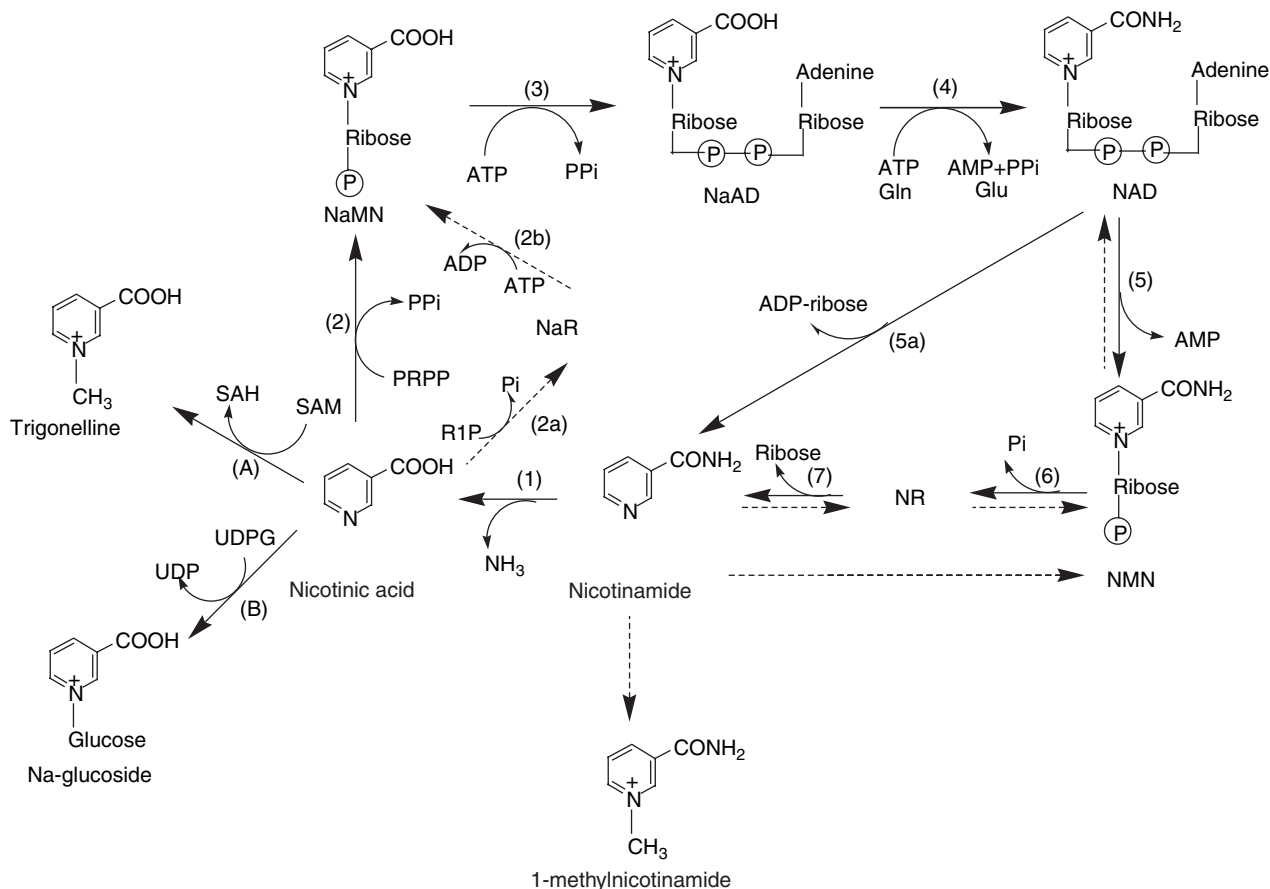


Fig. 1. Metabolic fate of nicotinamide in plants. PNC and synthesis of nicotinic acid conjugates are shown. A seven-member PNCVII (reactions 1–7), eight-member PNC VIII (reactions 1, 2a, 2b and 3–7) and five-member PNC V (reactions 1–4 and 5a) may be operative in plant cells. Other cycles, such as that including NR deaminase, are also possible. Trigonelline (reaction A) and Na-Glc (reaction B) are synthesised from nicotinic acid. PRPP, 5-phosphoribosyl pyrophosphate; R1P, ribose-1-phosphate; SAH, s-adenosyl-homocystein; SAM, s-adenosyl-methionine.

but their work has not been followed up nicotinamide is converted to 1*N*-methylnicotinamide in some organisms (Shibata et al. 1996), but no such reaction has been reported in plants. In some plant species, nicotinic acid 1*N*-glucoside (Na-Glc) is formed (Upmeier et al. 1988c). This compound has been incorrectly reported as nicotinic acid arabinoside (Willeke et al. 1979), but it was corrected as Na-Glc by the same group (Barz 1985, Upmeier et al. 1988c). Diversity of trigonelline and Na-Glc in plant cell cultures has been reported (Willeke et al. 1979).

Recently, some model plants have been used for metabolic studies of higher plants at molecular and/or cellular level. *Arabidopsis thaliana* is the most popular dicot model plant for which the genome has been fully sequenced. Rice (*Oryza sativa*) is used as a monocot model plant and has one of the smallest genomes of cereal species. *Lotus japonicus* is a legume model and is useful for studies of symbiosis and nitrogen fixation (Sato and Tabata 2006, Udvardi et al. 2005); its genome has

been partially sequenced. The tobacco (*Nicotiana tabacum*) Bright Yellow (BY)-2 cell line is often used in plant physiological studies at the cellular level (Nagata et al. 1992).

To understand the function of metabolic pathways in relation to physiological phenomena such as environment stress and growth stages of plants, databases on genome (DNA), transcriptome (mRNA), proteome (enzyme) and metabolome (metabolite) information are useful. If basic information on in situ metabolism is provided, such databases can be used more properly. This study seeks to determine the metabolic fate of ¹⁴C-labelled nicotinamide in cells and tissues of various plants, including model plants.

In addition to the model plants specified above, we examined the metabolic fate of [¹⁴C]nicotinamide in each organ of cacao seedlings. We also investigated trigonelline accumulation in various seeds and compared the metabolism of nicotinamide in seedlings of plants that store a high concentration of trigonelline in seeds (clover and alfalfa) and in plants that lack trigonelline in seeds

(chrysanthemum and radish). Our results suggest that nicotinamide salvage for NAD synthesis is generally active in actively growing cells of all plant species examined, but synthesis of trigonelline is absent in some plant species. Trigonelline synthesis was extremely high in leaves of rice, *L. japonicus*, cacao and radish, but no or little biosynthetic activity was detected in *A. thaliana* or cultured tobacco BY-2 cells. In contrast to trigonelline synthesis, Na-Glc formation was found in only a few plant species.

Materials and methods

Plant materials

Lotus japonicus seeds were obtained from the National Bioresource Project (*L. japonicus* and *Glycine max*) Core Facility Office, Department of Agriculture, Miyazaki University, Miyazaki, Japan. Seeds of *Picea glauca* were supplied by Professor E. C. Yeung, Department of Biological Sciences, University of Calgary, Canada. Seeds of rice (*O. sativa* cv. Nihonbare) were donated by Professor N. Yamamoto of our Department. Cacao seeds were supplied by Dr C. Nagai, Hawaii Agriculture Research Center, USA. Other seeds were purchased from Carolina Biological Supply Company, Burlington, NC, USA, or Sakata Seed Corporation, Yokohama, Japan. Seeds of *A. thaliana* accession Columbia and cell cultures of *A. thaliana*, rice, *L. japonicus* and tobacco BY-2 were obtained from the Experimental Plant Division of the RIKEN Bioresource Center. One-month-old *L. japonicus* plants with nodules (Oka-Kira et al. 2005) were kindly supplied by Dr M. Kawaguchi, University of Tokyo.

Tracer experiments with ^{14}C -labelled compounds

[Carbonyl- ^{14}C]nicotinamide (specific activity $1.96 \text{ GBq mmol}^{-1}$) and [carboxyl- ^{14}C]nicotinic acid (specific activity $1.92 \text{ GBq mmol}^{-1}$) were obtained from Moravex Biochemicals, Inc., Brea, CA.

We administered labelled compounds into the segments of plant tissues or into cultured cells according to the procedures of Zheng and Ashihara (2004) and Ashihara et al. (2005). Sample tissues or cells (approximately 100 mg FW) and 2 ml of 10 mM sucrose in 20 mM sodium phosphate buffer (pH 5.6), or 2 ml of the culture media specific for the materials, were placed in the main compartment of a 30-ml Erlenmeyer flask. The flask was fitted with a glass tube that contained a piece of filter paper impregnated with 0.1 ml of 20% KOH in a centre well. Each reaction was started by adding 10 μl of a solution of labelled compound (37 kBq) to the main compartment of the flask. The flasks were incubated in an oscillating water bath at 27°C, except for *A. thaliana*

(22°C). After incubation, the glass tube was removed from the centre well and placed in a 50-ml Erlenmeyer flask containing 10 ml of distilled water. Samples were collected by filtering through a tea strainer or a layer of Miracloth (Merck, Darmstadt, Germany) on a Büchner funnel, were washed with distilled water, then frozen with liquid nitrogen and stored at -80°C until extraction. Potassium bicarbonate that had been absorbed by the filter paper was allowed to diffuse into 10 ml distilled water overnight, and aliquots of the resulting solution (usually 0.5 ml) were used for the determination of radioactivity. Radioactivity was measured with a liquid scintillation analyser (Beckman, type LS 6500, Beckman Instruments, Fullerton, CA).

The labelled metabolites were extracted with 80% methanol (v/v) containing 20 mM sodium diethyldithiocarbamate and were analysed as described previously (Zheng and Ashihara 2004). Plant materials were homogenized in a mortar and pestle with the extraction medium, and the resulting methanol-soluble fraction was evaporated in vacuo to dryness at 37°C.

The labelled metabolites were separated by TLC using microcrystalline cellulose TLC plates (Merck, Darmstadt, Germany). Solvent systems I and IV of our previous paper (Zheng and Ashihara 2004) were used to identify the radiolabelled metabolites. Radioactivity on the TLC sheet was determined using a Bio-Imaging Analyser (Type FLA-2000; Fuji Photo Film Co., Ltd, Tokyo, Japan).

Determination of trigonelline content

Trigonelline was extracted with 80% (v/v) methanol containing 20 mM sodium diethyldithiocarbamate. After the methanol-soluble fraction had been evaporated in vacuo and dissolved in distilled water, the sample was analysed with HPLC, as in our previous paper (Zheng and Ashihara 2004) except that the absorbance was monitored using a Shimadzu Diode Array Detector, type SPD-M10A (Shimadzu Corp., Kyoto, Japan).

Results

Simple TLC analysis of ^{14}C -metabolites

In situ examination of the metabolic fate of [^{14}C]nicotinamide was performed with TLC. Fig. 2 shows a typical example of the separation of labelled metabolites by TLC. Most labelled compounds were efficiently separated by solvent I (*n*-butanol/acetic acid/water, 4/1/2, v/v) (Zheng and Ashihara 2004). However, nicotinic acid and nicotinamide appeared in a single spot, and separation of NAD and NADP, and also of NMN and NaMN, was not always clear. To overcome this problem, solvent IV (isobutyric acid/ammonia/water, 660/17/330) (Zheng and Ashihara 2004) was used. Spots corresponding to nicotinic

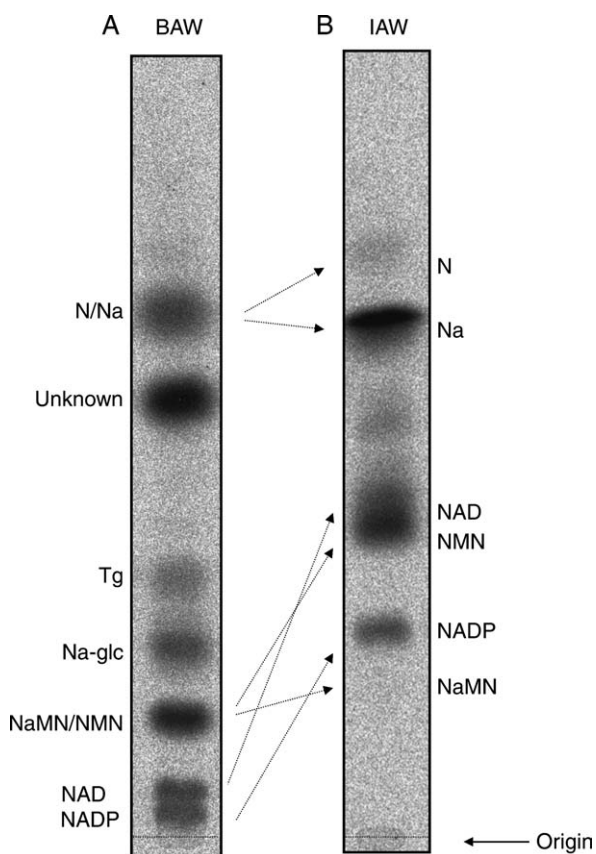


Fig. 2. Separation of ^{14}C -labelled metabolites by TLC. The profiles of ^{14}C -metabolites shown in this figure are from experiments with leaves of 50-day-old *Arabidopsis thaliana* accession Columbia, grown in a plant growth room at 22°C, 16:8 h of light : dark cycle. [Carbonyl- ^{14}C]-nicotinamide was administered to the leaf segments and incubated for 4 h. Solvent systems used for TLC are (A) *n*-butanol/acetic acid/water (BAW) (4/1/2) and (B) isobutyric acid/ammonia/water (IAW) (66/0/17/330). N, nicotinamide; Na, nicotinic acid; Tg, trigonelline.

acid and nicotinamide, and to NMN and NaMN, were clearly separated, and NADP was separated from other compounds including NAD by this system. We used this solvent system whenever it was necessary to separate these compounds. The TLC profiles shown in Fig. 2 were obtained from the methanol-soluble metabolites from *Arabidopsis* leaves. In addition to spots representing NADP, NAD, NaMN, NMN, Na-Glc, trigonelline, nicotinic acid and nicotinamide, a spot of unknown compound was observed in TLC with solvent I. This compound may be a metabolite unique to *A. thaliana*, since this spot is not observed in extracts from other plant materials.

Metabolism of [^{14}C]nicotinamide in *Arabidopsis thaliana*

Fig. 3 shows the metabolic fate of ^{14}C at 18 h after administration of [carbonyl- ^{14}C]nicotinamide in 50-day-

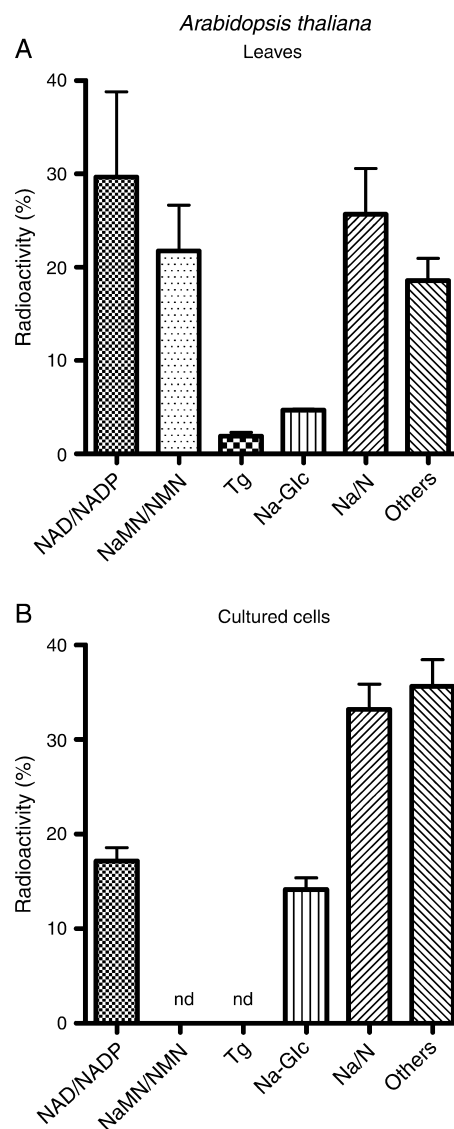


Fig. 3. Metabolic fate of [^{14}C -carbonyl]nicotinamide in (A) leaves and (B) cultured cells of *Arabidopsis thaliana* accession Columbia. The leaves are the same as those in Fig. 2. Suspension-cultured cells of *A. thaliana* (T87 strain) obtained from RIKEN were transferred to 0.7% agar gel containing Murashige–Skoog medium and were cultured for 20 days in the growth room. The metabolic fate of 10 μM [carbonyl- ^{14}C]nicotinamide (37 kBq) was analysed at 18 h after administration. Values are expressed as a percentage of radioactivity taken up by the samples and standard deviations ($n = 4$). nd, not detected; N, nicotinamide; Na, nicotinic acid; Tg, trigonelline.

old leaves (A) and 20-day-old cultured cells (B) of *A. thaliana* accession Columbia. In the leaves, radioactivity was found in the NAD/NADP, NaMN/NMN and Na/N fractions. The results of our preliminary experiments indicated that major components of the latter two fractions were NMN and nicotinic acid (data not shown). Only 4.7% of total radioactivity was recovered in Na-Glc

and 1.9% was recovered in trigonelline. In cultured cells, less than 20% of total radioactivity was found in NAD/NADP, and significant radioactivity was found in nicotinic acid and unidentified compounds. Nearly 15% of radioactivity was recovered in Na-Glc, but no radioactivity was found in trigonelline.

These results suggest that nicotinamide is readily converted to nicotinic acid and is salvaged for the NAD and NADP biosynthesis. Limited amounts of nicotinic acid are converted to nicotinic acid conjugates. Na-Glc is formed in both leaves and cultured cells. Very low biosynthetic activity of trigonelline was detected in leaves. In contrast to most other plant materials, radioactivity was recovered in unidentified compound(s) in *A. thaliana*, as shown in Fig. 2.

Metabolism of [¹⁴C]nicotinamide in rice

We examined the metabolic fate of radioactivity from [carbonyl-¹⁴C]nicotinamide using leaves of 14-day-old seedlings (Fig. 4A) and 20-day-old cultured cells (Fig. 4B) of rice. Large amounts of radioactivity were recovered in trigonelline 18 h after administration of labelled nicotinamide in leaves and in cultured cells. Considerable amounts (25–30%) of radioactivity were recovered in NAD/NADP, but little radioactivity was found in Na/N. No radioactivity was detected in Na-Glc. It follows that nicotinamide is efficiently used by rice leaves and by cultured rice cells for the synthesis of NAD and NADP and trigonelline.

Metabolism of [¹⁴C]nicotinamide in *Lotus japonicus*

The metabolic fate of [¹⁴C]nicotinamide in leaves of seedlings and in cultured cells of *L. japonicus* is shown in Fig. 5A, B. Major products were pyridine nucleotides and trigonelline in both materials, although more radioactivity was incorporated into trigonelline than into nucleotides in leaves; the reverse was found in cultured cells. Metabolism of [¹⁴C]nicotinamide was also compared in nodules, roots and leaves of 1-month-old *L. japonicus* plants (Fig. 6A–C). Almost all radioactivity from [¹⁴C]nicotinamide was recovered in the salvage products (NAD, NADP and NMN) in nodules (Fig. 6A). In roots and leaves, however, incorporation of ¹⁴C into these salvage products was only 20% of the total radioactivity (Fig. 6B, C). In leaves, as in the leaves of seedlings (Fig. 5A), a large amount of radioactivity was found in trigonelline (Fig. 6A). These results suggest that, in actively growing and undifferentiated tissues such as nodules, nicotinamide is used in the regeneration of NAD and NADP; in other words, an active pyridine nucleotide cycle (PNC) is

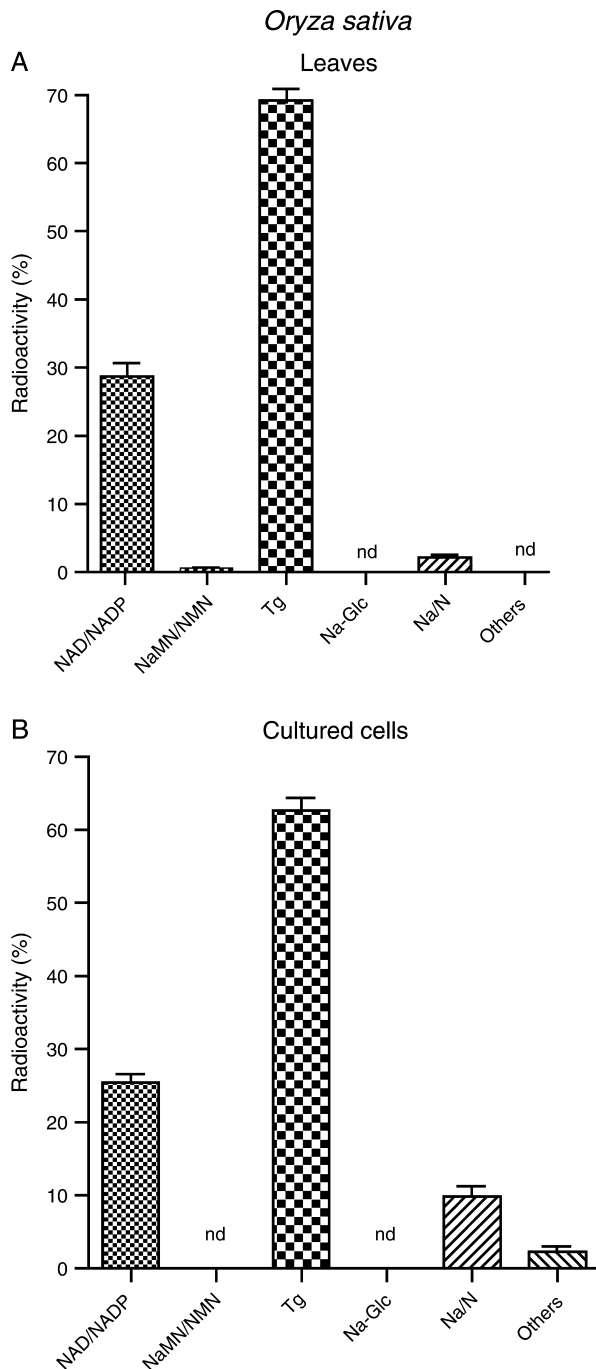


Fig. 4. Metabolic fate of [¹⁴C-carbonyl]nicotinamide in (A) leaves and (B) cultured cells of *Oryza sativa*. The leaves were taken from 14-day-old rice seedlings. Suspension-cultured rice cells were obtained from RIKEN and transferred to 0.7% agar gel containing Murashige–Skoog medium and were cultured for 20 days at 26°C in the dark. The metabolic fate of 10 μM [carbonyl-¹⁴C]nicotinamide (37 kBq) was analysed at 18 h after administration. Values, standard deviations and abbreviations of compound names are the same as shown in Fig. 3. nd, not detected.

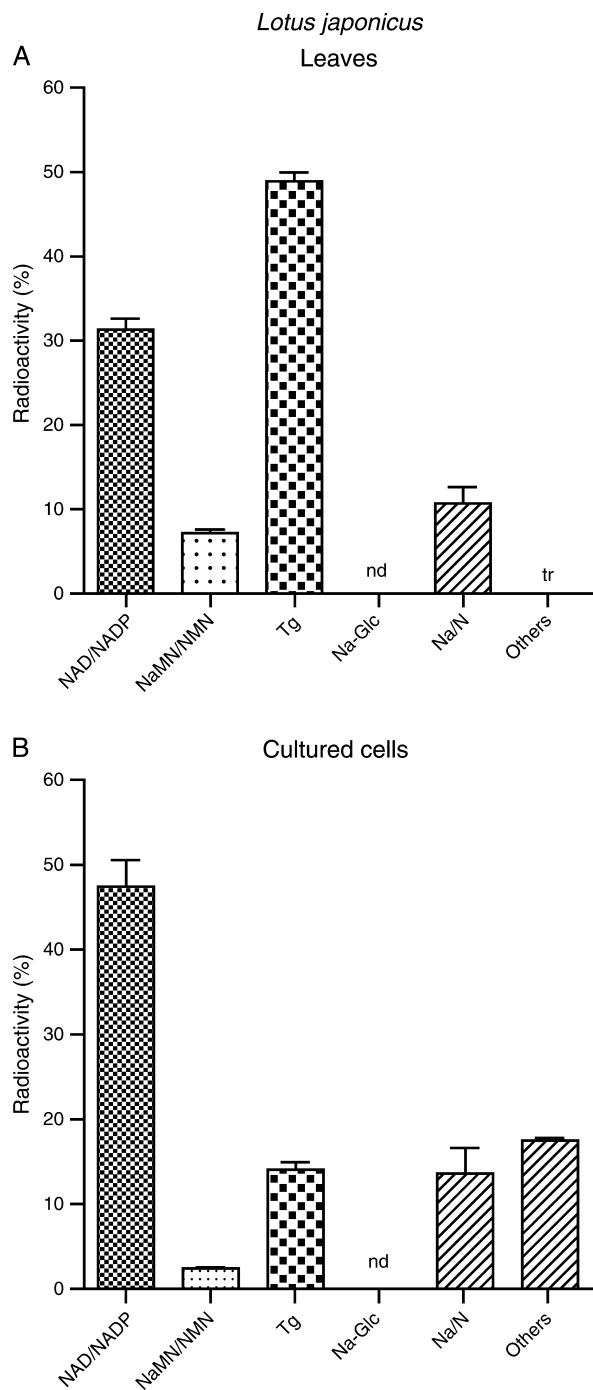


Fig. 5. Metabolic fate of [^{14}C -carbonyl]nicotinamide in (A) leaves and (B) cultured cells of *Lotus japonicus*. The leaves were taken from 7-day-old *L. japonicus* accession Miyakojima MG-20 seedlings grown in 0.55% agar gel in a light (18 h)/dark (6 h) cycle at a light intensity of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 22°C . Suspension-cultured *L. japonicus* cells obtained from RIKEN were transferred to 0.9% agar gel containing Murashige–Skoog medium and were cultured for 1 month in the dark at 26°C . The metabolic fate of $10 \mu\text{M}$ [carbonyl- ^{14}C]nicotinamide (37 kBq) was analysed at 18 h after administration. Values, standard deviations and abbreviations of compound names are the same as shown in Fig. 3. nd, not detected; tr, trace.

operating in these tissues. In contrast, in leaves, which are differentiated tissue, trigonelline synthesis is abundant.

Nicotinamide metabolism in tobacco BY-2 cells

The metabolic fate of [carbonyl- ^{14}C]nicotinamide in *N. tabacum* is shown in Fig. 7. Almost half of the radioactivity was found in Na-Glc, and up to 20% of radioactivity was recovered in the NAD/NADP fraction. No trigonelline was detected. Essentially, the same metabolic profile was obtained when [carbonyl- ^{14}C]nicotinamide was replaced by [carboxyl- ^{14}C]nicotinic acid (data not shown), so that nicotinamide seems to be easily converted to nicotinic acid and metabolised by the same routes as [^{14}C]nicotinic acid.

Nicotinamide metabolism in cacao seedlings

We compared nicotinamide metabolism in different parts of cacao seedlings (Fig. 8). In leaves, a major product of [carbonyl- ^{14}C]nicotinamide metabolism was trigonelline, but in taproots, both trigonelline and Na-Glc were formed. Conversion of [^{14}C]nicotinamide to salvage products (NAD, NADP and NMN) was greater in cotyledons than in other parts.

Survey of trigonelline accumulation in seeds from various plant species

We surveyed the concentration of trigonelline in seeds of various plant species; the results are shown in Table 1. Accumulation of trigonelline took place in Leguminosae seeds. The highest concentration (58 mM) was found in *Trifolium incarnatum*. In the Leguminosae seeds examined, the lowest value (0.9 mM) was found in *L. japonicus*. Smaller amounts of trigonelline were also found in seeds of rye, lettuce, sunflower and tomato.

Nicotinamide metabolism in seedlings grown from trigonelline-accumulating and non-trigonelline-accumulating seeds

As shown in Table 1, trigonelline is accumulated in seeds of certain plant species. In cotyledons and embryonic axes isolated from seedlings of the trigonelline-accumulating Leguminosae species, clover and alfalfa, a large amount of radioactivity from [^{14}C]nicotinamide was found in trigonelline and a lesser amount was recovered in the salvage products, but no radioactivity was found in Na-Glc (Fig. 9A, B). In a pair of the first leaves and the shoot–root axis of the non-trigonelline-accumulating plant garland chrysanthemum, both trig-

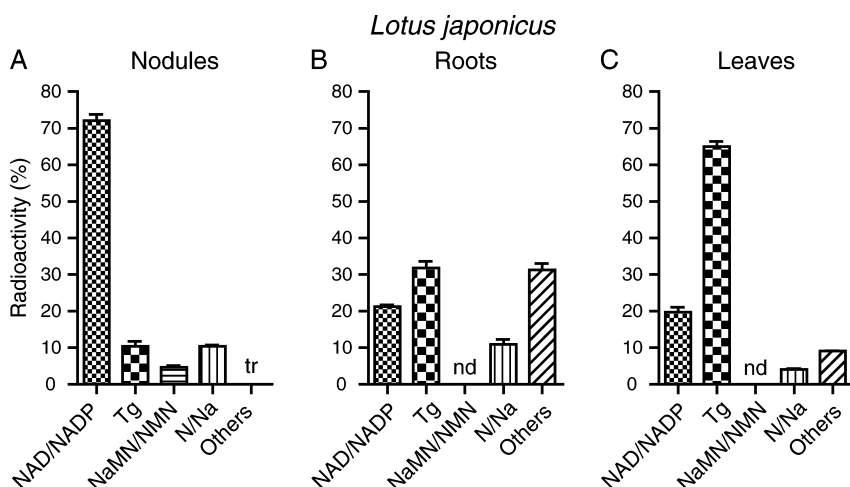


Fig. 6. Comparison of the metabolic fate of [^{14}C -carbonyl]nicotinamide in (A) nodules, (B) roots and (C) leaves of 1-month-old *Lotus japonicus* plants. The plants were placed under a light : dark cycle of 16:8 h at a light intensity of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 22°C . The metabolic fate of $10 \mu\text{M}$ [carbonyl- ^{14}C]nicotinamide (37 kBq) was analysed at 18 h after administration. Values, standard deviations and abbreviations of compound names are as shown in Fig. 3. nd, not detected; tr, trace.

onelline and Na-Glc were formed from [^{14}C]nicotinamide (Fig. 9C). In contrast, trigonelline alone was produced in germinating non-trigonelline-accumulating radish seeds (Fig. 9D). These results suggest that trigonelline-accumulating seeds have active trigonelline synthetic machinery in both cotyledons and embryonic axes. Even in non-trigonelline-accumulating seeds, trigonelline is produced during germination.

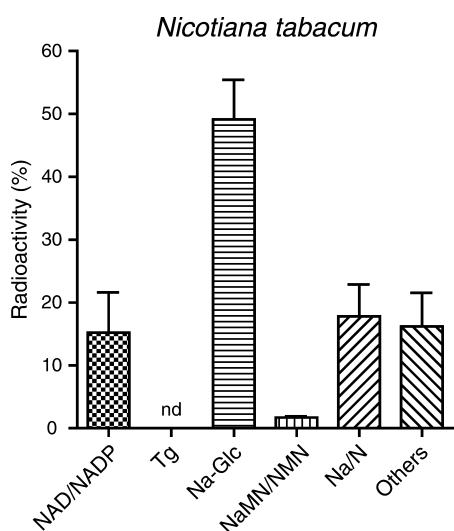


Fig. 7. Metabolic fate of [^{14}C -carbonyl]nicotinamide in cultured cells of *Nicotiana tabacum* BY-2. Suspension-cultured cells of tobacco BY-2 obtained from RIKEN were transferred to 0.7% agar gel containing Murashige–Skoog medium and were cultured for 20 days in the dark at 26°C . The metabolic fate of $10 \mu\text{M}$ [carbonyl- ^{14}C]nicotinamide (37 kBq) was analysed at 18 h after administration. Values, standard deviations and abbreviations of compound names are as shown in Fig. 3. nd, not detected.

Discussion

In plants, NAD is synthesized from nicotinamide and nicotinic acid by a salvage pathway that involves nicotinamidase (EC 3.5.1.19) and nicotinic acid phosphoribosyltransferase, but nicotinamide is not directly converted to pyridine nucleotides (Ashihara et al. 2005, Katoh and Hashimoto 2004). Therefore, as in coffee leaves and fruits (Zheng et al. 2004a) and mungbean seedlings (Zheng et al. 2005), NAD seems to begin the seven- or eight-component PNC (PNC VII or VIII), $\text{NAD} \rightarrow \text{NMN} \rightarrow \text{NR} \rightarrow \text{N} \rightarrow \text{NA} (\rightarrow \text{NaR}) \text{NaMN} \rightarrow \text{NaAD} \rightarrow \text{NAD}$ in plants. A few putative enzymes of the cycles have been found in the nucleotide databases of *A. thaliana* and *rice* (Katoh and Hashimoto 2004), namely nicotinamidase (step 1 in Fig. 1), nicotinate phosphoribosyl transferase (step 2) and NAD synthetase (step 4).

The present results suggest that plants may be grouped into three types according to the synthetic ability of nicotinic acid conjugates: trigonelline-forming plants (type I), Na-Glc-forming plants (type II) and plants forming both conjugates (type III). These differences seem to be because of the presence or expression of *S*-adenosylmethionine : nicotinic acid *N*-methyltransferase (step A in Fig. 1) and UDP glucose : nicotinic acid glucosyltransferase (step B). Although the presence of enzymes involved in these steps has been reported (Chen and Wood 2004, Upmeier et al. 1988a, 1988b, Zheng et al. 2005), no gene has been isolated from any species. Trigonelline accumulation in plants has been often reported; in contrast, Na-Glc seems to be only transiently accumulated (Barz 1985, Zheng et al. 2004a).

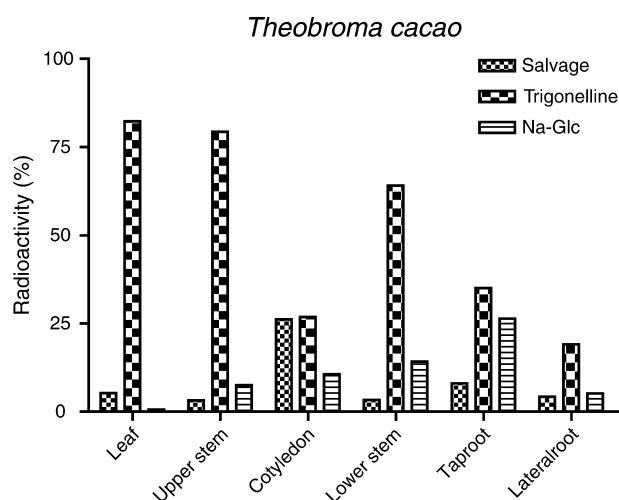


Fig. 8. Metabolic fate of [¹⁴C-carbonyl]nicotinamide in *Theobroma cacao* seedlings. Leaves, upper stem, cotyledons, lower stem, taproot and lateral roots were isolated from 1-month-old seedlings grown in a pot under natural conditions. The metabolic fate of 10 μ M [carbonyl-¹⁴C]nicotinamide (37 kBq) was analysed at 18 h after administration. Values and standard deviations are as shown in Fig. 3.

The functions of trigonelline in plants have been summarized by Minorsky (2002). They include cell cycle regulation, nodulation, oxidative stress and UV stress, DNA methylation, salt stress and nystinasty. However, the detailed molecular mechanisms are not yet known. The most plausible and simple function of trigonelline synthesis from nicotinic acid would be detoxification of excess nicotinic acid and nicotinamide released from the NAD cycle in cells. Exogenously supplied nicotinic acid and nicotinamide inhibited the growth of mungbean seedlings, but trigonelline has no such effect (Zheng et al. 2005). The role of trigonelline that has accumulated in seeds is not yet known. In mungbeans, trigonelline was transported from cotyledons to embryonic axis during germination but was not used in the salvage synthesis of NAD (Zheng et al. 2005). Although we could not confirm the conversion of trigonelline to nicotinic acid in our plant materials, trigonelline demethylating enzyme activities were reported by Taguchi and Shimabayashi (1983).

In contrast to trigonelline, Na-Glc seems to be easily metabolized. For example, in roots of tea seedlings, more than 50% of radioactivity from [carbonyl-¹⁴C]nicotinamide was recovered in Na-Glc during the first hour, but the rate of incorporation into Na-Glc was reduced to less

Table 1. Trigonelline contents in seeds

| English name | Scientific name | Family | Content (μ mol g ⁻¹ FW) |
|-----------------------|---------------------------------|---------------|---|
| Clover (Crimson) | <i>Trifolium incarnatum</i> | Leguminosae | 58.0 \pm 0.5 |
| Coffee | <i>Coffea arabica</i> | Rubiaceae | 52.5 \pm 0.8 |
| Alfalfa | <i>Medicago sativa</i> | Leguminosae | 12.2 \pm 1.1 |
| Kidney bean | <i>Phaseolus vulgaris</i> | Leguminosae | 6.2 \pm 0.7 |
| Mungbean | <i>Phaseolus aureus</i> | Leguminosae | 6.2 \pm 0.7 |
| Pea (Alaska) | <i>Pisum sativum</i> | Leguminosae | 2.7 \pm 0.3 |
| Horse bean | <i>Canavalia ensiformis</i> | Leguminosae | 2.2 \pm 0.3 |
| Soybean | <i>Glycine max</i> | Leguminosae | 1.2 \pm 0.3 |
| Miyakogusa trefoil | <i>Lotus japonicus</i> | Leguminosae | 0.9 \pm 0.1 |
| Rye | <i>Lolium perenne</i> | Gramineae | 0.40 \pm 0.09 |
| Lettuce | <i>Lactuca sativa</i> | Compositae | 0.30 \pm 0.05 |
| Sunflower | <i>Helianthus annuus</i> | Compositae | 0.09 \pm 0.06 |
| Rice | <i>Oryza sativa</i> | Gramineae | 0.04 \pm 0.00 |
| Tomato | <i>Lycopersicon esculentum</i> | Solanaceae | 0.03 \pm 0.02 |
| Wheat | <i>Triticum aestivum</i> | Gramineae | 0 |
| Maize | <i>Zea mays</i> | Gramineae | 0 |
| Oat | <i>Avena sativa</i> | Gramineae | 0 |
| Big quaking grass | <i>Briza maxima</i> | Gramineae | 0 |
| Stipa | <i>Stipa tenuissima</i> | Gramineae | 0 |
| Peppermint | <i>Mentha piperata</i> | Labiatae | 0 |
| Carrot | <i>Daucus carota</i> | Umbelliferae | 0 |
| Garland chrysanthemum | <i>Chrysanthemum coronarium</i> | Compositae | 0 |
| Radish | <i>Raphanus sativus</i> | Brassicaceae | 0 |
| Indian mustard | <i>Brassica juncea</i> | Brassicaceae | 0 |
| Field pumpkin | <i>Cucurbita pepo</i> | Cucurbitaceae | 0 |
| Cucumber | <i>Cucumis sativus</i> | Cucurbitaceae | 0 |
| Loblolly pine | <i>Pinus taeda</i> | Pinaceae | 0 |
| White spruce | <i>Picea glauca</i> | Pinaceae | 0 |

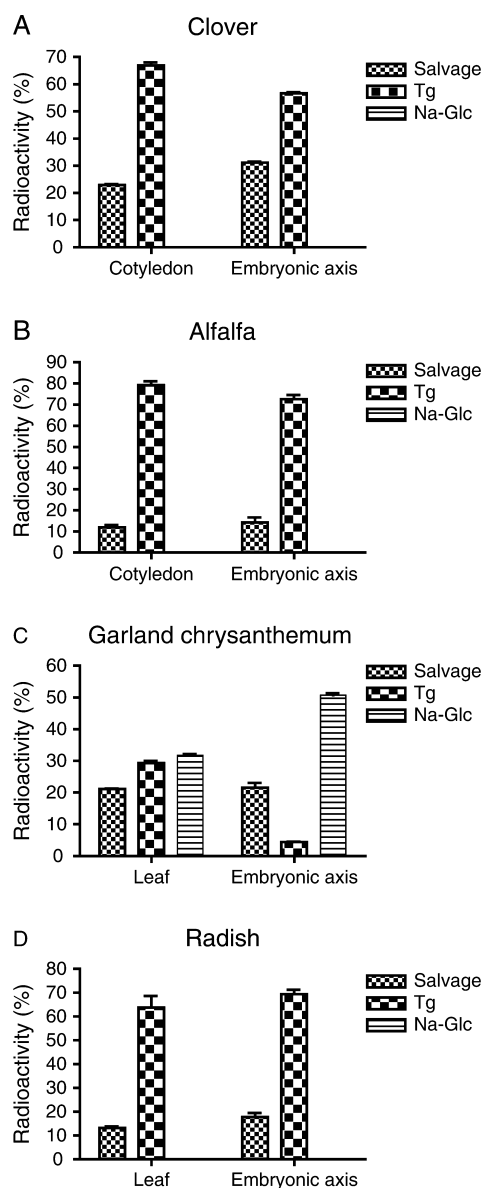


Fig. 9. Metabolic fate of [^{14}C -carbonyl]nicotinamide in cotyledons and embryonic axis of (A) *Trifolium incarnatum* (clover) and (B) *Medicago sativa* (alfalfa) and in primary leaves and embryonic axis (stem and root) of (C) *Chrysanthemum caronarium* (garland chrysanthemum) and (D) *Raphanus sativus* (radish). Samples were taken from 3-day-old seedlings grown in a sterilized 0.55% agar gel under natural conditions. The metabolic fate of 10 μM [carbonyl- ^{14}C]nicotinamide (37 kBq) was analysed at 18 h after administration. Values and standard deviations are as shown in Fig. 3. Tg, trigonelline.

than 15% by the following 17 h (Zheng et al. 2004). In the present work, the incubation time in most experiments was 18 h, so that conversion of nicotinamide to Na-Glc appears to be underestimated.

Based on the present study, we conclude that nicotinamide and nicotinic acid are involved in the plant PNC.

Nicotinamidase characterises the unique PNC in plants, which is distinguishable from the cycle in animals. After nicotinamide has been converted to nicotinic acid by this enzyme, it is simultaneously used for the synthesis of pyridine nucleotides. In plants, the activity of nicotinamidase is extremely high (Ashihara et al. 2005, Zheng et al. 2005), so that exogenously supplied nicotinamide is rapidly deaminated and nicotinic acid is produced. Recently, importance of nicotinamidase for plant NAD salvage was also argued by Wang and Pichersky (2007). Large amounts of nicotinic acid are converted to the nicotinic acid conjugates trigonelline and Na-Glc in plants when nicotinamide is supplied exogenously. Although we cannot exclude the possibility that this is caused artificially as a result of the supply of excess nicotinamide, plant pyridine metabolism can be characterized according to the formation of trigonelline and/or Na-Glc. The detailed metabolic role of these pyridine conjugates remains to be determined.

Acknowledgements – We thank Dr M. Kawaguchi of the University of Tokyo for advice and for providing *L. japonicus* plants.

References

- Ashihara H, Crozier A (1999) Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. *Adv Bot Res* 30: 118–205
- Ashihara H, Stasolla C, Yin Y, Loukanina N, Thorpe TA (2005) *De novo* and salvage biosynthetic pathways of pyridine nucleotides and nicotinic acid conjugates in cultured plant cells. *Plant Sci* 169: 107–114
- Barz W (1985) Metabolism and degradation of nicotinic acid in plant cell cultures. In: Neumann KH, Barz W, Reinhard E (eds) *Primary and Secondary Metabolism of Plant Cell Cultures*. Springer-Verlag, Berlin, pp 186–195
- Brenner C (2005) Evolution of NAD biosynthetic enzymes. *Structure* 13: 1239–1240
- Chen X, Wood AJ (2004) Purification and characterization of *S*-adenosyl-L-methionine nicotinic acid-*N*-methyltransferase from leaves of *Glycine max*. *Biol Plant* 48: 531–535
- Katoh A, Hashimoto T (2004) Molecular biology of pyridine nucleotide and nicotine biosynthesis. *Front Biosci* 9: 1577–1586
- Magni G, Amici A, Emanuelli M, Raffaelli N, Ruggieri S (1999) Enzymology of NAD $^{+}$ synthesis. *Adv Enzymol Relat Areas Mol Biol* 73: 135–182
- Minorsky PV (2002) Trigonelline: a diverse regulator in plants. *Plant Physiol* 128: 7–8
- Moat AG, Foster JW (1987) Biosynthesis and salvage pathways of pyridine nucleotides. In: Dolphin D, Avramovic O, Poulson R (eds) *Pyridine Nucleotide Coenzymes*. Chemical, Biochemical, and Medical Aspects, Part B. John Wiley & Sons, New York, pp 1–24

- Moffatt BA, Ashihara H (2002) Purine and pyrimidine nucleotide synthesis and metabolism. In: Somerville CR, Meyerowitz EM (eds) *The Arabidopsis Book*. American Society of Plant Biologists, Rockville, pp 1–20. doi: 10.1199/tab.0018. Available at www.aspb.org/publications/arabidopsis (accessed 1 July 2007)
- Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the 'HeLa' cell in the cell biology of higher plants. *Int Rev Cytol* 132: 1–30
- Noctor G, Queval G, Gakiere B (2006) NAD(P) synthesis and pyridine nucleotide cycling in plants and their potential importance in stress conditions. *J Exp Bot* 57: 1603–1620
- Oka-Kira E, Tateno K, Miura Ki, Haga T, Hayashi M, Harada K, Sato S, Tabata S, Shikazono N, Tanaka A, Watanabe Y, Fukuhara I, Nagata T, Kawaguchi M (2005) *klavier* (*klv*), a novel hypernodulation mutant of *Lotus japonicus* affected in vascular tissue organization and floral induction. *Plant J* 44: 505–515
- Revollo JR, Grimm AA, Imai SI (2004) The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *J Biol Chem* 279: 50754–50763
- Sato S, Tabata S (2006) *Lotus japonicus* as a platform for legume research. *Curr Opin Plant Biol* 9: 128–132
- Sestini S, Jacomelli G, Pescaglioni M, Micheli V, Pompucci G (2000) Enzyme activities leading to NAD synthesis in human lymphocytes. *Arch Biochem Biophys* 379: 277–282
- Shibata K, Shimada H, Taguchi H (1996) Fate of nicotinamide differs due to an intake of nicotinamide. *Biosci Biotechnol Biochem* 60: 1204–1206
- Stasolla C, Katahira R, Thorpe TA, Ashihara H (2003) Purine and pyrimidine nucleotide metabolism in higher plants. *J Plant Physiol* 160: 1271–1295
- Taguchi H, Shimabayashi Y (1983) Findings of trigonelline demethylating enzyme activity in various organisms and some properties of the enzyme from hog liver. *Biochem Biophys Res Commun* 113: 569–574
- Tramontano WA, Lynn DG, Evans LS (1983) Nicotinic acid and nicotinamide metabolism and promotion of cell arrest in G2 in *Pisum sativum*. *Phytochemistry* 22: 343–346
- Udvardi MK, Tabata S, Parniske M, Stougaard J (2005) *Lotus japonicus*: legume research in the fast lane. *Trends Plant Sci* 10: 222–228
- Upmeier B, Gross W, Koster S, Barz W (1988a) Purification and properties of S-adenosyl-L-methionine:nicotinic acid-N-methyltransferase from cell suspension cultures of *Glycine max* L. *Arch Biochem Biophys* 262: 445–454
- Upmeier B, Thomzik JE, Barz W (1988b) Enzymatic studies on the reversible synthesis of nicotinic acid-N-glucoside in heterotrophic parsley cell suspension cultures. *Z Naturforsch* 43c: 835–842
- Upmeier B, Thomzik JE, Barz W (1988c) Nicotinic acid-N-glucoside in heterotrophic parsley cell suspension cultures. *Phytochemistry* 27: 3489–3493
- Waller GR, Yang KS, Gholson RK, Hadwiger LA, Chaykin S (1966) The pyridine nucleotide cycle and its role in the biosynthesis of ricinine by *Ricinus communis* L. *J Biol Chem* 241: 4411–4418
- Wang G, Pichersky E (2007) Nicotinamidase participates in the salvage pathway of NAD biosynthesis in *Arabidopsis*. *Plant J* 49: 1020–1029
- Willeke U, Heeger V, Meise M, Neuhann H, Schindelmeiser I, Vordemfelde K, Barz W (1979) Mutually exclusive occurrence and metabolism of trigonelline and nicotinic acid arabinoside in plant cell cultures. *Phytochemistry* 18: 105–110
- Zheng XQ, Ashihara H (2004) Distribution, biosynthesis and function of purine and pyridine alkaloids in *Coffea arabica* seedlings. *Plant Sci* 166: 807–813
- Zheng XQ, Nagai C, Ashihara H (2004) Pyridine nucleotide cycle and trigonelline (N-methylnicotinic acid) synthesis in developing leaves and fruits of *Coffea arabica*. *Physiol Plant* 122: 404–411
- Zheng XQ, Hayashibe E, Ashihara H (2005) Changes in trigonelline (N-methylnicotinic acid) content and nicotinic acid metabolism during germination of mungbean (*Phaseolus aureus*) seeds. *J Exp Bot* 56: 1615–1623
- Zrenner R, Stitt M, Sonnewald U, Boldt R (2006) Pyrimidine and purine biosynthesis and degradation in plants. *Annu Rev Plant Biol* 57: 805–836