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Biosynthesis of trigonelline from nicotinate mononucleotide in mungbean seedlings

Xin-Qiang Zheng [a,b], Ayu Matsui [a], Hiroshi Ashihara [a,*]

[a] *Department of Biological Sciences, Graduate School of Humanities and Sciences, Ochanomizu University, Bunkyo-ku, Tokyo 112-8610, Japan*
[b] *Tea Research Institute, Zhejiang University, Kaixuan Road, Hangzhou 310029, China*

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Abstract

To determine the biosynthetic pathway to trigonelline, the metabolism of [carboxyl-^{14}C]nicotinate mononucleotide (NaMN) and [carboxyl-^{14}C]nicotinate riboside (NaR) in protein extracts and tissues of embryonic axes from germinating mungbeans (*Phaseolus aureus*) was investigated. In crude cell-free protein extracts, in the presence of *S*-adenosyl-L-methionine, radioactivity from [^{14}C]NaMN was incorporated into NaR, nicotinate and trigonelline. Activities of NaMN nucleotidase, NaR nucleosidase and trigonelline synthase were also observed in the extracts. Exogenously supplied [^{14}C]NaR, taken up by embryonic axes segments, was readily converted to nicotinate and trigonelline. It is concluded that the NaMN → NaR → nicotinate → trigonelline pathway is operative in the embryonic axes of mungbean seedlings. This result suggests that trigonelline is synthesised not only from NAD but also via the *de novo* biosynthetic pathway of pyridine nucleotides.

Keywords: Mungbean; *Phaseolus aureus*; Leguminosae; Biosynthesis; Pyridine alkaloid; Trigonelline

1. Introduction

Trigonelline (*N*-methylnicotinate) (**5**) (see Fig. 1) is a secondary metabolite formed from nicotinate (**4**) (Joshi and Handler, 1960). In many legumes and some species of other plant families, such as coffee (*Coffea* spp.), large amounts of trigonelline (**5**) (1–60 μmol/g fresh weight) accumulate in seeds (Koshiro et al., 2006; Matsui et al., 2007). Several physiological functions of trigonelline (**5**) have been proposed (Tramontano et al., 1982; Lynn et al., 1984; Ueda et al., 1995; Tramontano and Jouve, 1997; Wood, 1999; Minorsky, 2002; Zheng and Ashihara, 2004), but the biosynthesis and degradation of this alkaloid has not been fully elucidated. Since nicotinate (**4**) is derived from the degradation of NAD (**7**), it has been proposed that trigonelline (**5**) is a product of the pyridine nucleotide cycle originating from the pyridine nucleotides NAD and NADP (Zheng et al., 2004; Ashihara et al., 2005; Ashihara, 2006; Matsui et al., 2007). However, it is not clear that trigonelline (**5**) is synthesised exclusively by this pathway in plants. Direct nicotinate (**4**) formation from NaMN (**2**) initiated by *de novo* nucleotide biosynthesis is also plausible. The direct route from NaMN (**2**) to nicotinate (**4**) in nicotine-producing tobacco roots has been proposed by Wagner et al. (1986), who suggested that NaMN glycohydrolase contributes to this conversion. However, the K_m value for NaMN (**2**) of NaMN glycohydrolase was extremely high (4 mM). The cellular concentration of NaMN (**2**) in mungbean seedlings is so low that it is difficult to detect using HPLC (X.-Q. Zheng, unpublished observation). Even if the pathway from NaMN (**2**) to nicotinate (**4**) is operative, other enzymes may therefore participate in this process in trigonelline (**5**)-forming

Abbreviations: IMP, inosine-5′-mononucleotide; NaAD, nicotinate adenine nucleotide; NaMN, nicotinate mononucleotide; NaR, nicotinate riboside; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; PPi, pyrophosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate.

* Corresponding author. Tel./fax: +81 3 5978 5358.
E-mail address: ashihara.hiroshi@ocha.ac.jp (H. Ashihara).

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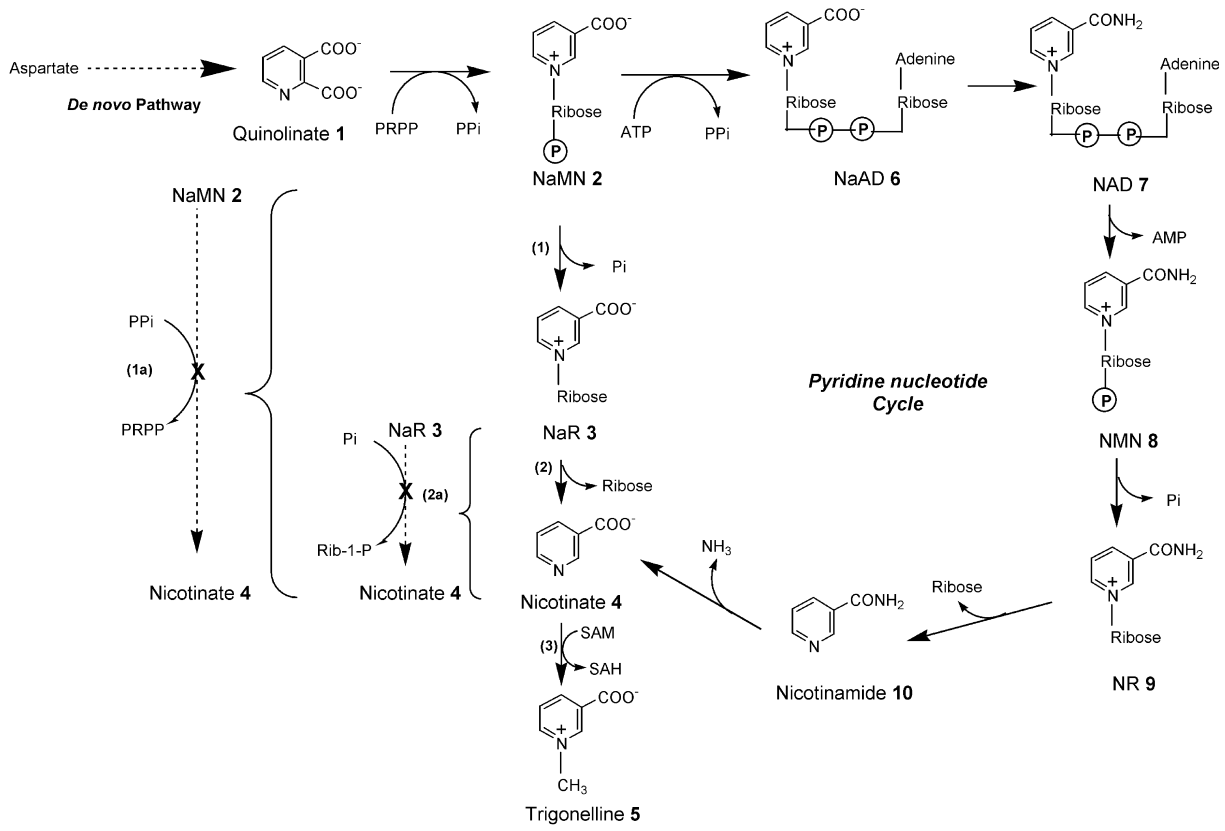

Fig. 1. Proposed pathway of trigonelline (**5**) synthesis from NaMN (**2**). Steps (1)–(3) are respectively, catalysed by NaMN nucleotidase, NaR nucleosidase and trigonelline synthase. The alternative enzymes, nicotinate phosphoribosyltransferase or NaMN glycohydrolase (reaction is not shown), might participate in the step (1a), and nucleoside phosphorylase in step (2a), although no evidence was obtained here. Trigonelline (**5**) synthesis via the pyridine nucleotide cycle is also shown.

mungbean plants. Here, we report experimental results that support formation of trigonelline (**5**) from NaMN (**2**) via NaR (**3**) in mungbean embryonic axes. The classic pathway of trigonelline (**5**) synthesis via pyridine nucleotide cycle, and the new proposed route are both illustrated in Fig. 1.

2. Results and discussion

2.1. Conversion of NaMN (2) to trigonelline (5) in vitro

It has been reported that trigonelline (**5**) is produced from the degradation of NAD (**7**) via the pyridine nucleotide cycle (Zheng et al., 2004, 2005; see Fig. 1). However, there is no evidence that trigonelline (**5**) is formed exclusively by this route. It is also speculated that nicotinate (**4**), which is a direct precursor of trigonelline (**5**), is also derived from NaMN (**2**) itself newly synthesised by the *de novo* pathway. To demonstrate this hypothesis, we first examined the conversion of NaMN (2) to trigonelline (**5**) in the enzyme extracts *in vitro*.

Fig. 2 shows the time-course of the *in vitro* metabolism of [carboxyl-^{14}C]NaMN (**2**) in the presence of 1 mM SAM and dialysed protein extracts from embryonic axes of 3-day-old mungbean seedlings. Two min after adminis-

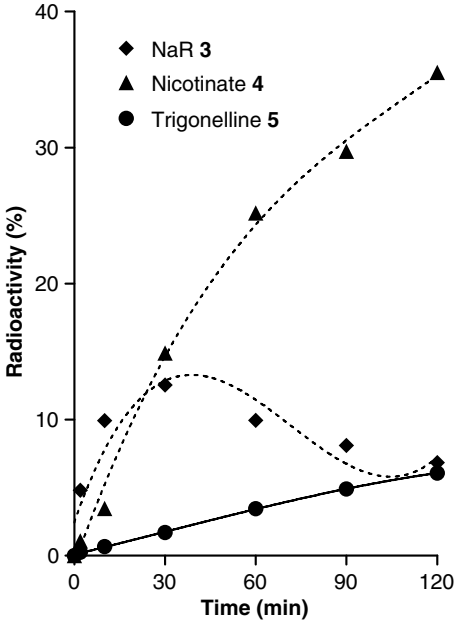

Fig. 2. Metabolic fate of 18 μM [^{14}C]NaMN (2) (3.7 kBq) in the presence of 1 mM SAM and 10 mM $MgCl_2$ in soluble protein extracts (320 μg) from embryonic axes taken from 3-day-old mungbean seedlings. Values are expressed as a percentage of total radioactivity (3.7 kBq) and SD ($n = 3$) in the reaction mixture. The rest of radioactivity was recovered in unmetabolised NaMN (2).

tration of [^{14}C]NaMN (**2**), radioactivity was incorporated into NaR (3) (ca. 4.8% of radioactivity administered). After 10 min, radioactivity was also with associated nicotinate (**4**) and trigonelline (**5**). This finding suggests that NaR (**3**) is the initial metabolite of NaMN (**2**). The radioactivity in NaR (**3**) increased transiently up to 30 min and then decreased. After 30 min, the most heavily labelled compound was nicotinate (4) (14.9–35.5% of radioactivity administered). As expected, radioactivity from [^{14}C]NaMN (**2**) was detected in trigonelline (**5**), and it increased linearly with incubation time. The direct pathway from NaMN (**2**) to trigonelline (**5**) via NaR (**3**) and nicotinate (**4**) therefore functions at least in the crude protein extracts from mungbean embryonic axes. Conversion of NaMN (**2**) to NaR (**3**) is catalysed by nucleotidase(s), and conversion of NaR (**3**) to nicotinate (**4**) is hydrolysed by nucleosidase(s), although we do not know if NaMN (**2**) specific-nucleotidase and NaR (**3**) specific-nucleosidase are present in mungbean extracts.

2.2. Metabolic fate of [^{14}C]NaMN (2) in vitro

NaMN (**2**) has a crucial role in pyridine metabolism (Fig. 1). In addition to the conversion to NaR (**3**), NaMN (**2**) is converted to NaAD (**6**) by NaMN adenylyltransferase (EC 2.7.7.18) and utilised for NAD (**7**) synthesis in many organisms including plants (Katoh and Hashimoto, 2004; Noctor et al., 2006; Hashida et al., 2007). Therefore, we examined the *in vitro* metabolic fate of [^{14}C]NaMN (2) in the presence of 1 mM ATP, another substrate of NaMN adenylyltransferase (Fig. 3). 2 min after addition of [^{14}C]NaMN (**2**), 2.7%, 2.5% and 0.7% of administered NaMN (**2**) was converted to NaAD (**6**), NaR (**3**) and nicotinate (**4**), respectively. However, after longer incubation, NaAD (**6**) was the predominant metabolite and it comprised ca. 27% of total activity after 30 min. The rest of the radioactivity was retained in NaMN (**2**). The data suggest that NaMN (**2**) is preferentially used for NaAD (**6**) synthesis if sufficient ATP is provided, with a smaller amount of NaMN (**2**) being converted to NaR (**3**) and nicotinate (**4**).

2.3. Activity of enzymes involved in NaMN (2) metabolism

The activities of NaMN adenylyltransferase, NaMN nucleotidase, NaR nucleosidase and trigonelline synthase were measured individually using [^{14}C]NaMN (**2**), [^{14}C]NaR (**3**) and [^{14}C]nicotinate (**4**) as substrates, as shown in Table 1. NaMN adenylyltransferase had the highest activity and NaR nucleosidase activity was about 1.5 times that of the NaMN nucleotidase activity.

In addition to direct hydrolytic cleavage, the conversion of NaMN (**2**) to nicotinate (**4**) can, in theory, be catalysed by the reverse reaction of nicotinate phosphoribosyltransferase (Fig. 1, reaction 1a). However, this pathway appears to be negligible in mungbean extracts, as up to 1 mM PPi did not stimulate the conversion. Participation of nucleoside phosphorylase (Fig. 1, reaction 2a) in the conversion of NaR (**3**) to nicotinate (**4**) is not feasible, because Pi inhibits the conversion of NaR (**3**) to nicotinate (**4**) (data not shown). These reactions therefore appear not to be involved in the conversion of NaMN (**2**) to nicotinate (**4**). Involvement of NaMN glycohydrolase in this conversion, which has been suggested to occur in tobacco roots (Wagner et al., 1986), cannot be wholly excluded, because radioactivity in NaMN (**2**) was incorporated into nicotinate (**4**); however, the rapid incorporation of radioactivity into NaR (**3**) was always observed so that the two-step conversion catalysed by nucleotidase and nucleosidase appears to be the major route.

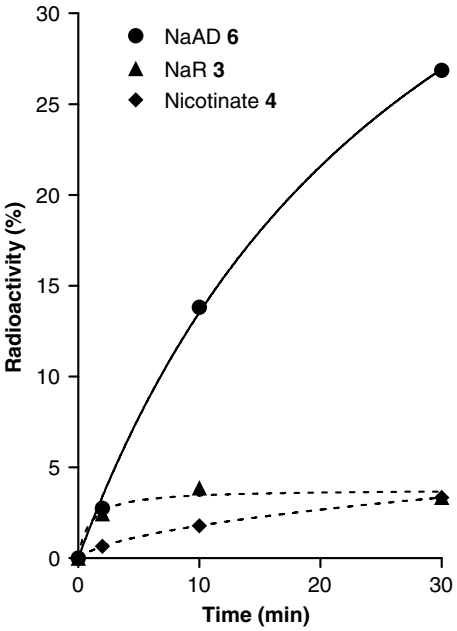

Fig. 3. Metabolic fate of 18 μM [^{14}C]NaMN (2) (3.7 kBq) in the presence of 1 mM ATP and 10 mM $MgCl_2$ in soluble protein extracts (320 μg) from embryonic axes taken from 3-day-old mungbean seedlings. Values are expressed as a percentage of total radioactivity (3.7 kBq) and SD ($n = 3$) in the reaction mixture. The rest of radioactivity was recovered in unmetabolised NaMN (**2**).

Table 1
Activities of enzymes involved in NaMN (**2**) metabolism

Enzyme name	EC number	Activity
NaMN adenylyltransferase	2.7.7.18	40.6
NaMN nucleotidase	Not registered	11.6
NaR nucleosidase	Not registered	16.7
Trigonelline synthase	2.1.1.7	3.2

Enzyme activities are expressed as pkat per mg protein.
The values show average of the activity obtained from two separate experiments.

2.4. Uptake of [carboxyl-^{14}C]NaMN (2)

The *in vitro* enzyme activity strongly suggests the conversion of NaMN (**2**) to trigonelline (**5**) occurs in a way which bypasses the pyridine nucleotide cycle. However,

results obtained from *in vitro* experiments do not always reflect metabolism *in vivo* because enzymes are sometimes located in different intracellular compartments. We therefore examined *in situ* metabolism of 9 μM [carboxyl-^{14}C]NaMN (2) (37 kBq) in segments of 3-day-old mungbean embryonic axes. Almost no uptake of [^{14}C]NaMN (**2**) by the embryonic axes was observed, and no radioactive metabolites were detected in methanol-soluble materials (data not shown). This is probably because the transport system of NaMN (**2**) is not present in mungbean cell membranes.

2.5. In situ metabolism of [carboxyl-^{14}C] NaR (3)

In contrast to [^{14}C]NaMN (2), approximately 2% and 4% of the administered 10 μM [carboxyl-^{14}C] NaR (3) (37 kBq) had been taken up by segments of 3-day-old mungbean embryonic axes at 2 h and 4 h after incubation, respectively. The radioactivity was distributed in nucleotides (mainly NaMN (**2**), NAD (**7**) and NADP), trigonelline (**5**), nicotinate (**4**) and unmetabolised NaR (**3**) (Fig. 4). The radioactivity in nucleotides and trigonelline (**5**) increased with time, but radioactivity in nicotinate (**4**) and NaR (**3**) was almost constant. These results suggest that NaR (**3**) was not only utilised for nucleotide synthesis, but also for trigonelline (**5**) synthesis. Although NaR (**3**) has not yet been isolated from any organism, incorporation of the radioactivity from [carbonyl-^{14}C]nicotinamide (**10**) into NaR (**3**) in leaves of *Coffea arabica* has been observed (Zheng et al., 2004). The endogenous concentration of NaMN (**2**) in mungbean seedlings may be too low to detect using ordinary HPLC methods.

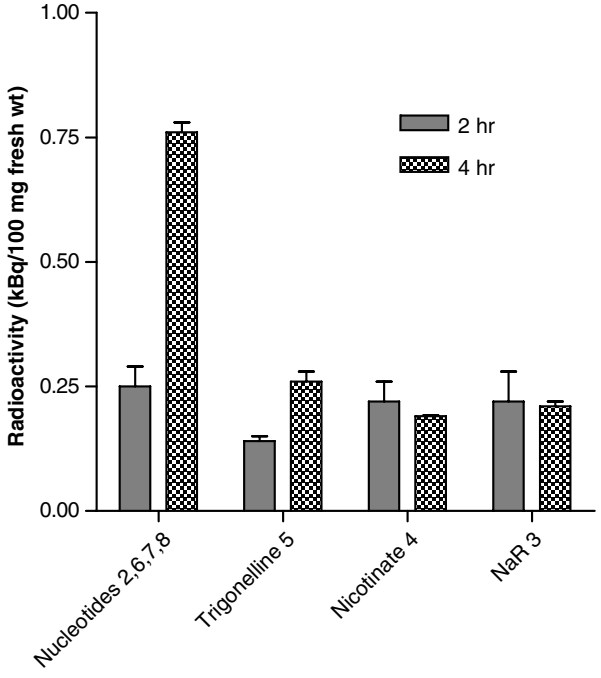

Fig. 4. *In situ* metabolism of 10 μM [^{14}C] NaR (3) (37 kBq) in embryonic axes of 3-day-old mungbean seedlings. Values are expressed as a percentage of total uptake of radioactivity and SD ($n = 3$). Total uptake of [^{14}C] NaR (**3**) by the embryonic axes (100 mg fresh weight) was 0.83 ± 0.15 kBq (2h) and 1.42 ± 0.03 kBq (4h).

2.6. Concluding remarks

Our results using crude extracts of mungbean seedlings and *in situ* tracer experiments with [^{14}C] NaR (**3**) suggest that part of NaMN (**2**) originating from *de novo* pyridine nucleotide synthesis is converted to nicotinate (**4**) via NaR (**3**) and used for trigonelline (**5**) synthesis. This is the first report that trigonelline (**5**) is synthesised directly from the *de novo* synthesis of NaMN (**2**) by three enzymatic systems, namely NaMN nucleotidase, NaR nucleosidase and trigonelline synthase. NaMN (**2**) produced by *de novo* biosynthesis appears to be utilised preferentially for NAD (**7**) synthesis, because NaMN adenylyltransferase activity is greater than the activity of NaMN nucleotidase. Overflow of NaMN (**2**) produced by the *de novo* pathway may be directed to trigonelline (**5**) synthesis.

3. Experimental

3.1. Chemicals

[Carboxyl-^{14}C]nicotinate mononucleotide (sp. act 2.0 GBq mmol^{-1}), [carboxyl-^{14}C]nicotinate riboside (sp. act 1.9 GBq mmol^{-1}) and [carboxyl-^{14}C]nicotinate (sp. act 1.9 GBq mmol^{-1}) were obtained from Moravek Biochemicals Inc, Brea, CA, USA. Biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

3.2. Plant materials

Mungbean seeds (*Phaseolus aureus* Roxb) were purchased from the Carolina Biological Supply Company, Burlington, NC, USA. Seeds sterilised with saturated NaOCl solution were germinated on 0.55% agar gel in Erlenmeyer flasks at 26 °C in the dark, as detailed in previous papers (Ashihara et al., 1974; Zheng et al., 2005). Embryonic axes were removed from cotyledons, and were promptly used for enzyme extraction and *in situ* ^{14}C-tracer experiments.

3.3. Extraction of soluble protein extracts

Embryonic axes (ca. 4 g fresh weight) were homogenised in extraction medium, consisting of 50 mM HEPES-NaOH buffer (pH 7.6), 2 mM sodium EDTA, 2 mM dithiothreitol and 5 mM $MgCl_2$, using a mortar and pestle. The homogenate was centrifuged at 20,000*g* for 20 min at 2 °C. The supernatant was treated with 80% satd. $(NH_4)_2SO_4$, and the precipitate was collected by centrifugation at 20,000*g* for 15 min. The resulting pellet was dissolved in 50 mM HEPES-NaOH buffer (pH 7.6) and desalted on a prepacked column of Sephadex G-25.

The protein content was determined by the method of Bradford (1976).

3.4. In vitro metabolism of NaMN (2) in soluble protein extracts

In vitro conversion of [^{14}C]NaMN (**2**) was examined in the presence of SAM or ATP in the soluble mungbean protein fraction that was precipitated with 80% saturated ammonium sulphate. The total volume of the reaction mixture was 100 μl, and incubation took place at 30 °C. The reaction mixture contained 30 mM HEPES-NaOH buffer (pH 7.6), 18 μM [^{14}C]NaMN (**2**) (3.7 kBq), 10 mM $MgCl_2$, desalted enzyme extracts and 1 mM SAM (or 1 mM ATP). Degradation of NaMN (**2**) was also examined using a reaction mixture consisting of 30 mM HEPES-NaOH buffer (pH 7.6), 18 μM [^{14}C]NaMN (**2**) (3.7 kBq), 10 mM $MgCl_2$ and desalted enzyme extracts. The reactions were terminated by transferring the test tubes to a boiling water bath for 2 min. After brief centrifuging, an aliquot of each supernatant was loaded onto the cellulose TLC plate. Labelled compounds were separated by TLC using the solvent system I (*n*-BuOH–HOAc–H_2O, 4:1:2 v/v) as described by Zheng and Ashihara (2004).

3.5. Determination of enzyme activity

The same crude protein fraction was used for individual enzyme assays. The total volume of the reaction mixture was 100 μl, and incubation took place at 30 °C. The mixture for NaMN adenylyltransferase contained 30 mM HEPES–NaOH buffer (pH 7.6), 18 μM [^{14}C]NaMN (2) (3.7 kBq), 1 mM ATP, 10 mM $MgCl_2$ and desalted enzyme extracts. The reaction mixtures for NaMN nucleotidase contained 30 mM HEPES–NaOH buffer (pH 7.6), 18 μM [^{14}C]NaMN (**2**) (3.7 kBq), 10 mM $MgCl_2$ and desalted enzyme extracts. The reaction mixture for NaR (**3**) nucleosidase was the same, except that NaMN (**2**) was replaced by 20 μM [^{14}C] NaR (**3**) (3.7 kBq). The reaction mixture for trigonelline synthase contained 30 mM HEPES–NaOH buffer (pH 7.6), 18 μM [^{14}C]nicotinate (3.7 kBq), 1 mM SAM, 10 mM $MgCl_2$ and desalted enzyme extracts. The reaction was performed in an oscillating water bath at 30 °C. The substrate and product were separated by TLC, as described above. The proportionality of reaction velocity to the amount of enzyme extracts was checked for every assay by plotting the initial velocity against at least three different amounts of enzymes. In the assay condition, the reaction velocity was constant for at least 5 min (NaMN adenylyltransferase, NaMN nucleotidase and NaR nucleosidase) or 30 min (trigonelline synthase) and was proportional to the amount of enzymes (0–200 mg protein).

3.6. In situ tracer experiments

The ^{14}C-labelled NaMN (**2**) and NaR (**3**) were administered to mungbean embryonic axes, as described in a previous paper (Ashihara et al., 2000, 2005). A plant sample (100 mg fresh weight) and 2.0 ml of 20 mM sodium phosphate buffer (pH 5.6) containing 10 mM sucrose were placed in the main compartment of a 30 ml Erlenmeyer flask. The flask was fitted with a small glass tube containing a piece of filter paper impregnated with 0.1 ml of 20% KOH in the centre well, to collect $^{14}CO_2$. Each reaction was started by adding a solution of radioactive compound (37 kBq) to the main compartment of the flask. The flasks were incubated in an oscillating water bath at 27 °C. After incubation, the plant materials were harvested, washed with distilled H_2O, kept in an extraction medium consisting of MeOH–H_2O (4:1, v/v) methanol with 20 mM sodium diethyldithiocarbamate, and stored at −30 °C prior to extraction.

Labelled metabolites were extracted and analysed as described previously (Zheng and Ashihara, 2004). In summary, segments of the embryonic axes were homogenised in a pestle and mortar with the extraction medium. The resulting MeOH-soluble fraction was concentrated using a rotary evaporator and was separated by TLC using microcrystalline cellulose TLC plates (Merck, Darmstadt, Germany). Solvent system I of the previous paper (Zheng and Ashihara, 2004) was used to identify radio-labelled metabolites.

3.7. Determination of radioactivity

Radioactivity was measured using a multi-purpose scintillation counter (type LS 6500, Beckman, Fullerton, CA, USA). The distribution of radioactivity on the TLC sheet was analysed using a Bio-Imaging Analyser (Type, FLA-2000, Fuji Photo Film Co., Ltd. Tokyo, Japan).

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References

Ashihara, H., 2006. Metabolism of alkaloids in coffee plants. Braz. J. Plant Physiol. 18, 1–8.

Ashihara, H., Komamine, A., Shimokoriyama, M., 1974. Glucose catabolism during ageing and differentiation in hypocotyls of Phaseolus mungo seedlings. Bot. Mag. Tokyo 87, 121–131.

Ashihara, H., Stasolla, C., Loukanina, N., Thorpe, T.A., 2000. Purine and pyrimidine metabolism in cultured white spruce (Picea glauca) cells: Metabolic fate of^{14} C-labeled precursors and activity of key enzymes. Physiol. Plant 108, 25–33.

Ashihara, H., Stasolla, C., Yin, Y., Loukanina, N., Thorpe, T.A., 2005. De novo and salvage biosynthetic pathways of pyridine nucleotides and nicotinic acid conjugates in cultured plant cells. Plant Sci. 169, 107–114.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Hashida, S., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., 2007. *Arabidopsis thaliana* nicotinate/nicotinamide mononucleotide adenyltransferase (AtNMNAT) is required for pollen tube growth. Plant J. 49, 694–703.

Joshi, J.G., Handler, P., 1960. Biosynthesis of trigonelline. J. Biol. Chem. 235, 2981–2983.

Katoh, A., Hashimoto, T., 2004. Molecular biology of pyridine nucleotide and nicotine biosynthesis. Front Biosci. 9, 1577–1586.

Koshiro, Y., Zheng, X.Q., Wang, M., Nagai, C., Ashihara, H., 2006. Changes in content and biosynthetic activity of caffeine and trigonelline during growth and ripening of *Coffea arabica* and *Coffea canephora* fruits. Plant Sci. 171, 242–250.

Lynn, D.G., Lewis, D.H., Tramontano, W.A., Evans, S., 1984. Induction of cell arrest in G2: structural specificity of trigonelline. Phytochemistry 23, 1225–1228.

Matsui, A., Yin, Y., Yamanaka, K., Iwasaki, M., Ashihara, H., 2007. Metabolic fate of nicotinamide in higher plants. Physiol. Plant., doi:10.1111/j.1399-3054.2007.00959.x.

Minorsky, P.V., 2002. Trigonelline: a diverse regulator in plants. Plant Physiol. 128, 7–8.

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.

Tramontano, W.A., Hartnett, C.M., Lynn, D.G., Evans, L.S., 1982. Relationship between trigonelline concentration and promotion of cell arrest in G2 in cultured roots of *Pisum sativum*. Phytochemistry 21, 1201–1206.

Tramontano, W.A., Jouve, D., 1997. Trigonelline accumulation in salt-stressed legumes and the role of other osmoregulators as cell cycle control agents. Phytochemistry 44, 1037–1040.

Ueda, M., Yamamura, S., Niwa, M., 1995. Trigonelline, a leaf-closing factor of the nyctinastic plant, *Aeschynomene indica*. Phytochemistry 39, 817–819.

Wagner, R., Feth, F., Wagner, K.G., 1986. The pyridine-nucleotide cycle in tobacco: enzyme activities for the recycling of NAD. Planta 167, 226–232.

Wood, A.J., 1999. Comparison of salt-induced osmotic adjustment and trigonelline accumulation in two soybean cultivars. Biol. Plant. (Prague) 42, 389–394.

Zheng, X.Q., 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, X.Q., Ashihara, H., 2004. Distribution, biosynthesis and function of purine and pyridine alkaloids in *Coffea arabica* seedlings. Plant Sci. 166, 807–813.

Zheng, X.Q., 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.