Changes in trigonelline (N-methylnicotinic acid) content and nicotinic acid metabolism during germination of mungbean (Phaseolus aureus) seeds

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Abstract

Changes in trigonelline content and in biosynthetic activity were determined in the cotyledons and embryonic axes of etiolated mungbean (Phaseolus aureus) seedlings during germination. Accumulation of trigonelline (c. 240 nmol per pair of cotyledons) was observed in the cotyledons of dry seeds; trigonelline content decreased 2 d after imbibition. Trigonelline content in the embryonic axes increased with seedling growth and reached a peak (c. 380 nmol per embryonic axis) at day 5. Trigonelline content did not change significantly during the differentiation of hypocotyls, and the concentration was greatest in the apical 5 mm. Nicotinic acid and nicotinamide were better precursors for pyridine nucleotide synthesis than quinolinic acid, but no great differences were found in the synthesis of trigonelline from these three precursors. Trigonelline synthase activity increased in the embryonic axes, but decreased in cotyledons during germination. [¹⁴C]Nicotinic acid and trigonelline absorbed by the cotyledons were transported to the embryonic axes during germination. Trigonelline had no effect on the growth of seedlings, but nicotinic acid and nicotinamide significantly inhibited the growth of roots. Based on these findings, the role of trigonelline synthesis in mungbean seedlings is discussed.

Key words: Detoxification, mungbean, NAD metabolism, nicotinamide, nicotinic acid, Phaseolus aureus, pyridine nucleotide cycle, seed germination, translocation, trigonelline.

Introduction

Metabolism of pyrimidine and purine nucleotides has been investigated by the present authors in leguminous seeds during germination (Ashihara, 1977, 1983; Nobusawa and Ashihara, 1983). Pyridine (nicotinamide adenine) nucleotides are also important nucleotides for life, because they are coenzymes for redox reactions. Many legumes produce trigonelline as a secondary metabolite derived from NAD. Several physiological functions of trigonelline have been proposed (Minorsky, 2002), but little is known about the biosynthesis and metabolism of this compound in plants. Using pea seedlings, Evans and co-workers suggested that trigonelline is a plant hormone present in cotyledons, and that it promotes cell arrest in G2 during cell maturation in roots and shoots (Evans et al., 1979; Tramontano et al., 1982; Evans and Tramontano, 1984).

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Materials and methods

Radiochemicals

\[^1^H\]Quinolinic acid (specific activity 200 GBq mmol\(^{-1}\)), [carboxyl-\[^1^4^C\]nicotinamide (specific activity 1.96 GBq mmol\(^{-1}\)), and [carboxyl-\[^1^4^C\]nicotinic acid (specific activity 1.92 GBq mmol\(^{-1}\)) were obtained from Moravek Biochemicals, Inc, Brea, CA, USA. [Carboxyl-\[^1^4^C\]trigonelline was prepared using a commercially available [carboxyl-\[^1^4^C\]nicotinic acid and mungbean cotyledons. In summary, slices of mungbean cotyledons (c. 2 g) were incubated with phosphate buffer and 9 MBq [carboxyl-\[^1^4^C\]nicotinic acid for 18 h. After extraction, the trigonelline was purified with a Dowex 1-X4 column (Cl\(^-\) form) and HPLC according to the methods detailed in a previous paper (Zheng and Ashihara, 2004), and its specific activity was 2.4 MBq mmol\(^{-1}\).

Plant materials

Seeds of mungbean (Phaseolus aureus Roxb=Vigna radiate L.) were obtained from the Carolina Biological Supply Company, Burlington, NC, USA. Eighty seeds were sterilized with a saturated solution of sodium hypochlorite and were placed on 100 ml of 0.55% agar in a 500 ml Erlenmeyer flask. The flasks were kept in the dark at 26 °C. Cotyledons and embryonic (shoot-root) axes were separated and used as experimental materials. To examine the effect of nicotinic acid, nicotinamide, and trigonelline on seedling growth, 1 mM solutions of these three compounds were sterilized using a disposable syringe filter unit (Advantec Toyo, Tokyo, Japan) and were added to the agar medium.

Determination of endogenous levels of trigonelline

Trigonelline was extracted with 80% methanol and analysed with HPLC, as in a previous paper (Zheng and Ashihara, 2004) except that the absorbance was monitored using a Shimadzu Diode Array Detector, type SPD-M10A.

Administration and analysis of labelled precursors

The labelled compounds were administered as described in a previous paper (Ashihara et al., 2000). Samples (c. 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 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\(_2\). For \[^1^H\]quinolinic acid, flasks without the centre well were used. Each reaction was started by adding a solution of radioactive compound 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 water, kept in an extraction medium (80% methanol containing 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). Plant materials were homogenized in a pestle and mortar with the extraction medium specified above, and the resulting methanol-soluble fraction was separated by TLC using microcrystalline cellulose TLC plates (Merck, Darmstadt, Germany). Solvent systems I and IV of the previous paper (Zheng and Ashihara, 2004) were used to identify radiolabelled metabolites. Radioactivity on the TLC sheet was determined using a Bio-Imaging Analyser (Type, FLA-2000, Fuji Photo Film Co., Ltd. Tokyo, Japan). Spots of \(^3^H\) on the TLC were scraped off and eluted from the cellulose support with water, and radioactivity was determined using a liquid scintillation counter (Beckman, type LS 6500).

Transport and metabolism of radiolabelled nicotinic acid and trigonelline

Sets of ten sterilized mungbean seeds were incubated with 1.2 ml (37 kBq) of solution of [carboxyl-\[^1^4^C\]nicotinic acid or [carboxyl-\[^1^4^C\]trigonelline. Absorption of radioactive compounds was completed after 6 h. After that, some of the seeds were germinated on 0.55% agar and others were immediately analysed. The cotyledons and embryonic axes of the 1-, 2-, 3-, and 4-d-old seedlings were collected and analysed.

Determination of enzyme activity

The activity of all enzymes in this study was determined using labelled substrates. The total volume of reaction mixtures was 100 µl and incubation took place at 30 °C. The enzyme reactions were terminated by transferring the test tubes to a boiling water bath. After brief centrifuging, the precipitate was removed and an aliquot of each sample was loaded onto the cellulose TLC plate. Labelled substrate and product were separated by TLC using the same solvent as described above. All reaction mixtures for enzyme assay contained 50 mM HEPES-NaOH buffer (pH 7.6) together with the following components. (i) Quinolinate phosphoribosyltransferase: 0.05 mM [\[^1^H\]quinolinic acid (specific activity, 3.6 GBq mmol\(^{-1}\)), 0.75 mM PRPP, 10 mM MgCl\(_2\), and 1 mM DTT. (ii) Nicotinamide phosphoribosyltransferase and nicotinamide phosphoribosyltransferases: the components were the same as quinolinate phosphoribosyltransferase, but 0.05 mM quinolinic acid was replaced by [carboxyl-\[^1^4^C\]nicotinic acid (specific activity, 310 MBq mmol\(^{-1}\)) or [carboxyl-\[^1^4^C\]nicotinamide (specific activity, 310 MBq mmol\(^{-1}\)). (iii) Nicotinamidase: 0.1 mM [carboxyl-\[^1^4^C\]nicotinic acid (specific activity, 170 MBq mmol\(^{-1}\)). (iv) Trigonelline synthase: 0.1 mM [carboxyl-\[^1^4^C\]nicotinic acid (specific activity, 170 MBq mmol\(^{-1}\)), 1 mM S-adenosyl-L-methionine.

Results

Changes in endogenous trigonelline content during germination

Figure 1A shows the fresh weight of a pair of cotyledons and an embryonic axis in various stages of germination of mungbean seedlings. The fresh weight of cotyledons increased for 1 d after imbibition and then decreased. The weight in the embryonic axis increased rapidly after 2 d. Similar growth has been reported of beans (Ashihara, 1977; Ashihara and Matsumura, 1977).

The changes in trigonelline content in organs and its concentration in cotyledons and embryonic axes during germination were determined (Fig. 1B, C). The seeds...
accumulated a high amount of trigonelline (c. 250 nmol seed\(^{-1}\)), and 96% of total trigonelline was distributed in the cotyledons. The trigonelline content in cotyledons fell rapidly after 3 d of germination. The concentration of trigonelline g\(^{-1}\) fresh weight was high in seeds, but decreased after 1 d, consistent with the water uptake. The trigonelline content in embryonic axes increased gradually and reached a peak (c. 380 nmol axis\(^{-1}\)) at day 5. Its concentration fell rapidly during the first few days.

**Distribution of trigonelline in different parts of the hypocotyl**

Hypocotyls of germinating mungbean seeds contain differing stages of cells (Ashihara and Sato, 1993). The hypocotyls (55 mm) of 4-d-old etiolated mungbean seedlings were separated into parts: part 1 (the apical 5 mm of the hypocotyls including hook), 2 (5–15 mm), 3 (15–25 mm), 4 (25–35 mm), 5 (35–45 mm), and 6 (45–55 mm). The cells in part 1 consisted of young cells, whereas elongation cells were distributed in parts 2 to 4, and parts 5 and 6 contained a large number of ageing cells (Ashihara et al., 1974). Trigonelline was distributed in all parts of the hypocotyl, although a high concentration was found in part I (Fig. 2).

**Metabolism of \[^{3}\text{H}\]quinolinic acid, \[^{14}\text{C}\]nicotinic acid, and \[^{14}\text{C}\]nicotinamide**

In coffee, \[^{3}\text{H}\]quinolinic acid, \[^{14}\text{C}\]nicotinic acid, and \[^{14}\text{C}\]nicotinamide were used as precursors for trigonelline synthesis (Zheng et al., 2004). To investigate which compound is the best precursor for trigonelline synthesis in mungbean, the metabolism of these compounds was...
compared using cotyledons and embryonic axes of 1-d-old seedlings (Fig. 3). In cotyledons, 50–60% of the radioactivity was recovered in trigonelline and 20% of the radioactivity from [3H]quinolinic acid; nearly 40% of that from [carbonyl-14C]nicotinamide and [carboxyl-14C]nicotinic acid was distributed in pyridine nucleotides. In embryonic axes, more than 70% of the radioactivity of the three precursors was incorporated into trigonelline, and 10% of the radioactivity from [3H]quinolinic acid and more than 20% of that from [carbonyl-14C]nicotinamide and [carboxyl-14C]nicotinic acid was distributed in the nucleotides. These results suggest that nicotinic acid mononucleotide is produced by both de novo and salvage pathways, and is then used for NAD and trigonelline synthesis in mungbean seedlings.

**Effect of concentration of nicotinic acid on its metabolism**

To investigate whether the concentration of nicotinic acid alters the metabolic fate of [carboxyl-14C]nicotinic acid, differing concentrations of nicotinic acid were administered to the embryonic axes of 2-d-old seedlings, and the distribution of radioactivity was examined (Table 1). When the segments of axes were incubated with 6–1000 μM [carboxyl-14C]nicotinic acid, the total uptake of nicotinic acid varied from 1.5 to 334 nmol (100 mg)–1 fresh weight. At a lower concentration range (6–15 μM), the rate of conversion from nicotinic acid to pyridine nucleotides was higher at 6 μM (c. 17% of total radioactivity) than at 15 μM (c. 5%), but that to trigonelline was similar for these two concentrations (78% and 74%, respectively). These results suggest that nicotinic acid is used preferentially for pyridine nucleotides synthesis, and the rest is for trigonelline synthesis. Trigonelline synthesis increased up to 30 nmol (100 mg)–1 fresh weight when the exogenous nicotinic acid concentration was increased to 1 mM. However, nearly 90% of the nicotinic acid taken up by the segments remained (Table 1).

**Changes in nicotinic acid metabolism in cotyledons and embryonic axes during germination**

In cotyledons, more than one half of [carboxyl-14C]nicotinic acid taken up by the segments was converted to pyridine nucleotides and less than a quarter of the total activity to trigonelline (Fig. 4). Nicotinic acid salvage activity for pyridine nucleotide synthesis was therefore higher than the biosynthetic activity of trigonelline. No significant metabolic changes were observed during germination. Interestingly, trigonelline synthesis still occurred even in the later stage of germination in which trigonelline content was markedly reduced. In the embryonic axes, there was much more trigonelline synthetic activity than pyridine nucleotide synthesis during the entire period of germination. Trigonelline synthesis fell gradually after 3 d of germination. This suggests that trigonelline synthesis is greater in young cells than in older ones.

**Translocation of trigonelline and related compounds in germinating seeds**

To examine the translocation of trigonelline that accumulated in cotyledons, [carboxyl-14C]nicotinic acid was administered for 6 h. Almost all the radioactivity was recovered from the cotyledons of the seeds; about 60% was distributed in trigonelline and 30% in pyridine nucleotides (Fig. 5A). These radioactive seeds were planted in agar medium, and germinated in the dark. After 1, 2, 3, and 4 d, the seedlings were collected and separated into cotyledons and embryonic axes. More than 80% of the radioactivity observed in the cotyledons of the seeds had moved to the embryonic axes with 4 d (Fig. 5A). The radioactivity of trigonelline in the cotyledons of imbibed seeds was c. 2.3 kBq, but that in embryonic axes at day 4 was 2.6 kBq (Fig. 6B). The radioactivity of trigonelline in cotyledons decreased gradually and reached a minimum of 0.4 kBq on day 4 (Fig. 6A).

In addition to trigonelline in imbibed seeds, some NAD and NADP in the seeds may therefore be converted to trigonelline and/or other degradation products and transported to the embryonic axes.

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Fig. 3. Comparison of metabolism of 0.5 μM [3H]quinolinic acid, 9.4 μM [carbonyl-14C]nicotinamide, and 9.6 μM [carboxyl-14C]nicotinic acid in cotyledons (A) and embryonic axes (B) of 1-d-old mungbean seedlings. The duration of incubation was 18 h. The rates of incorporation of radioactivity into various metabolites are expressed as a percentage of the total radioactivity taken up by the segments. Mean values and SD (n=3) are shown. NaMN/NMN, nicotinic acid mononucleotide plus nicotinamide mononucleotide; Na/N, nicotinic acid plus nicotinamide; Qa, quinolinic acid; Tg, trigonelline.
To confirm the translocation of trigonelline, $^{14}$C-trigonelline was prepared using the mungbean seedlings shown in the Materials and methods, and the same experiments were performed using [carboxyl-$^{14}$C]trigonelline (Fig. 5B). Trigonelline in cotyledons moved gradually to the embryonic axes, and $c. 80\%$ of the radioactivity of trigonelline was found in the axes on day 4. No conversion of trigonelline to other compounds was detected.

These results suggest that trigonelline stored in cotyledons is transported to the embryonic axes during germination. However, transported trigonelline is not easily metabolized, at least during germination in the dark.

**Effect of trigonelline and related compounds on the growth of seedlings**

The effect of exogenously supplied 1 mM trigonelline, nicotinamide, and nicotinic acid on the fresh weight of mungbean seedlings and on the length of shoots and roots was studied (Fig. 7A-C). Nicotinamide and nicotinic acid slightly inhibited the rise in fresh weight during germination, but trigonelline had no such effect. Remarkably, nicotinic acid and nicotinamide severely inhibited root growth (Fig. 7C). No significant effect of these compounds on the growth of shoots was observed, but trigonelline promoted shoot growth.

**Changes in activity of trigonelline metabolism enzymes**

Significant activities of quinolinate phosphoribosyltransferase, nicotinate phosphoribosyltransferase, and nicotinamide were found in cotyledons and embryonic axes, but no nicotinamide phosphoribosyltransferase activity was detected (Table 2). ATP is required for the activity of nicotinate phosphoribosyltransferase, but not for quinolinate phosphoribosyltransferase. Therefore, quinolinic acid and nicotinic acid were salvaged by the respective phosphoribosyltransferases directly, but nicotinamide appeared to be converted to nicotinic acid mononucleotide after conversion to nicotinic acid by nicotinate phosphoribosyltransferase. In fact, a small amount of $^{14}$C-nicotinic acid mononucleotide was detected when the nicotinamide phosphoribosyltransferase was assayed with $^{14}$C-nicotinamide (data not shown).

### Table 1. Effect of concentration of nicotinic acid on the metabolic fate of [carboxyl-$^{14}$C]nicotinic acid in embryonic axes of 2-d-old mungbean seedlings

<table>
<thead>
<tr>
<th>Concentration</th>
<th>6 μM</th>
<th>15 μM</th>
<th>100 μM</th>
<th>1000 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotides</td>
<td>1.26±0.00 (16.7)</td>
<td>1.24±0.01 (5.1)</td>
<td>0.54±0.14 (1.9)</td>
<td>2.96±0.50 (0.9)</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>1.20±0.02 (78.0)</td>
<td>3.38±0.52 (73.6)</td>
<td>7.17±2.46 (25.2)</td>
<td>29.72±0.16 (8.9)</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.07±0.01 (4.7)</td>
<td>0.98±0.49 (21.3)</td>
<td>20.73±6.60 (72.8)</td>
<td>301.25±1.10 (90.2)</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.01±0.00 (0.7)</td>
<td>0.00±0.00 (0.0)</td>
<td>0.04±0.01 (0.1)</td>
<td>0.13±0.03 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>1.54±0.05 (100)</td>
<td>4.60±1.12 (100)</td>
<td>28.48±1.13 (100)</td>
<td>334.06±1.00 (100)</td>
</tr>
</tbody>
</table>

Fig. 4. Metabolic fate of 9.6 μM [carboxyl-$^{14}$C]nicotinic acid (specific activity, 1.92 GBq mmol$^{-1}$) in cotyledons (A) and embryonic axes (B) of 1-, 3-, and 5-d-old mungbean seedlings. The duration of incubation was 4 h. The rates of incorporation of radioactivity into various metabolites are expressed as a percentage of the total radioactivity taken up by the segments. The mean values and SD ($n=3$) are shown. The nucleotides are NAD, NADP, and very small amounts of nicotinic acid mononucleotide and nicotinamide mononucleotide. Abbreviations, see legend for Fig. 3.
There was trigonelline synthase activity in dry seeds. This activity increased in the embryonic axes, but decreased in cotyledons during germination (Fig. 8). A similar pattern was found for quinolinate phosphoribosyltransferase and nicotinate phosphoribosyltransferase (data not shown). By contrast, nicotinamidase activity increased in both cotyledons and embryonic axes during germination (Fig. 8).

**Discussion**

Accumulation of trigonelline is found in some Leguminosae seeds, such as clover, alfalfa, and mungbean (X Zheng, Yamanaka, H Ashihara, unpublished observations). The present study suggests that trigonelline is formed from nicotinic acid derived from NAD.

In mungbean seedlings, NAD is synthesized by both the de novo and the salvage pathways, as in many other organisms (Magni et al., 1999; Sestini et al., 2000; Katoh and Hashimoto, 2004). In animals, nicotinamide is salvaged by nicotinamide phosphoribosyltransferase (Rocchigiani et al., 1992; Revollo et al., 2004), but in mungbean seedlings it is converted to nicotinic acid by nicotinamidase.
and a product, nicotinic acid, is salvaged by nicotinate phosphoribosyltransferase. Wagner et al. (1986) also reported that they could not find any nicotinamide phosphoribosyltransferase activity in tobacco extracts. As found in coffee leaves and fruits (Zheng et al., 2004), the six component pyridine nucleotide cycle (PNC VI)

\[
\text{NAD} \rightarrow \text{nicotinamide mononucleotide} \rightarrow \text{nicotinamide} \rightarrow \text{nicotinic acid} \rightarrow \text{nicotinic acid mononucleotide} \rightarrow \text{nicotinic acid adenine dinucleotide} \rightarrow \text{NAD}
\]

operates in both cotyledons and embryonic axes of germinating mungbean seeds. However, an enzyme, which catalyses the conversion of nicotinamide mononucleotide to nicotinamide riboside, was found to be active in mungbean extracts. Therefore, the seven-membered pyridine nucleotide cycle (PNC VII) could be operative in mungbean seedlings (Fig. 9).

Like other enzymes of glycolysis and pyrimidine biosynthesis (Ashihara and Kameyama, 1989; Ashihara and Matsumura, 1977), the activities of trigonelline synthase (Fig. 8) and enzymes related to pyridine nucleotide synthesis (data not shown) were found in extracts from dry mungbean seeds; their activities decreased with ageing of the cotyledons. By contrast, nicotinamidase activity was significant in cotyledons during germination (Fig. 8). A similar pattern has been reported for proteolytic and amylolytic enzymes (Minamikawa, 1979).

The present results suggest that trigonelline is translocated from cotyledons to embryonic axes, like other

### Table 2. Activities of enzymes related to pyridine nucleotide metabolism in mungbean seedlings

Enzyme extracts were obtained from 1-d-old seedlings. Enzyme activities are expressed as pkat mg⁻¹ protein. Values are means and SD obtained from three extracts, except for quinolinate phosphoribosyltransferase. nd, not detected.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cotyledons</th>
<th>Embryonic axes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinolinate phosphoribosyltransferase⁴</td>
<td>0.60</td>
<td>5.39</td>
</tr>
<tr>
<td>Nicotinate phosphoribosyltransferase⁴</td>
<td>0.47 ±0.04</td>
<td>1.68 ±0.17</td>
</tr>
<tr>
<td>Nicotinamide phosphoribosyltransferase⁶</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Nicotinamidase</td>
<td>0.92±0.05</td>
<td>3.47±0.34</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trigonelline synthase</td>
<td>1.29±0.07</td>
<td>3.15±0.54</td>
</tr>
</tbody>
</table>

⁴ Mean from two different extracts.

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**Fig. 7.** Effect of 1 mM trigonelline, 1 mM nicotinamide, and 1 mM nicotinic acid on the growth of mungbean seedlings during germination. The effect on the fresh weights of shoot-root axes (A) and effect on the length of shoot (B) and root (C) is shown. Fresh weights are expressed as mg. The length of shoots and roots is expressed as mm. The mean values and SD (n=10) are shown.
storage compounds, during germination. Since $^{14}$C-trigonelline administered to mungbean cotyledons was moved to embryonic axes, but was not converted to nicotinic acid (Fig. 5B), it is unlikely that trigonelline acts as a storage form of nicotinic acid, at least during germination. Although trigonelline demethylating activity has been found in extracts of some plant seeds (Shimizu and Mazzafera, 2000; Taguchi and Shimabayashi, 1983), these preliminary results suggest little or no activity of trigonelline dimethylase in extracts from mungbean seedlings. Shimizu and Mazzafera (2000) reported that trigonelline might act as a reserve molecule for NAD synthesis in coffee seeds, but only at the very early stages of germination or in specific tissues such as the embryo.

Exogenously supplied nicotinic acid and nicotinamide inhibited the growth of mungbean seedlings, but trigonelline has no such effect (Fig. 7). Therefore, one of the reasons for trigonelline synthesis in mungbean seedlings could be the detoxification of excess nicotinic acid and nicotinamide released from the NAD cycle in the cells.

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References


