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Biosynthesis of nicotianamine in the suspension-cultured cells of tobacco *(Nicotiana megalosiphon)*

Shinsuke Shojima[1], Naoko K. Nishizawa[1], Shinji Fushiya[2], Shigeo Nozoe[2], Takashi Kumashiro[3], Toshiyuki Nagata[4], Tomoko Ohata[1], and Satoshi Mori[1]

[1] Laboratory of Plant Nutrition and Fertilizers, Department of Agricultural Chemistry, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan
[2] Pharmaceutical Institute, Tohoku University, Sendai, Japan
[3] Iwata Experiment Station, Japan Tobacco Inc., Shizuoka, Japan
[4] Department of Cell Biology, National Institute for Basic Biology, Okazaki, Japan

Summary. Seven kinds of suspension cell cultures from five species of *Nicotiana* were screened for the occurrence of nicotianamine. Nicotianamine was detected in the cultured cells of *N. megalosiphon* and *N. plumbaginifolia*. L-[1-^{14}C]Methionine, which is the precursor of the mugineic-acid-family phytosiderophores and nicotianamine in barley plants, was incorporated into nicotianamine by the cultured cells of *N. megalosiphon* both in vivo and in vitro. The advantage of the cultured tobacco cells for the study of the biosynthesis of nicotianamine and the mugineic-acid-family phytosiderophores is discussed.

Key words: Nicotianamine — Phytosiderophore — Mugineic acid — Biosynthesis — *Nicotiana*

Introduction

Mugineic-acid-family phytosiderophores are iron-chelating compounds (Fig. 1) which are secreted by the roots of several grasses (Takagi 1976; Takemoto et al. 1978; Nomoto et al. 1981; Takagi et al. 1984; Mori et al. 1987; Kawai et al. 1988) and by the suspension-cultured cells of barley (Nishizawa et al. 1989) in response to iron-deficiency stress to solubilize and absorb the iron. To purify the biosynthetic enzymes, we recently developed a cell-free system for the biosynthesis of nicotianamine (Shojima et al. 1989), a putative precursor of the mugineic-acid-family phytosiderophores, from the substrate L-methionine (Mori and Nishizawa 1987). However, since our system is derived from the root tips of the iron-deficient barley plants, it is difficult to obtain sufficient material. Not more than 10 g fresh mass of the root tips can be obtained from the 60 iron-deficient barley plants at one month after germination.

Nicotianamine was first isolated from tobacco plants (Noma et al. 1971); it has been suggested that its function is to regulate the cellular transport and metabolism of ferrous or other divalent transition-metal ions by binding them (Scholz et al. 1985, 1988a, 1988b; Schreiber 1986). Although the biosynthetic pathway from L-methionine via azetidine-2-carboxylic acid and its dimeric compound [*N*-(3-amino-3-carboxypropyl)azetidine-2-carboxylic acid] to nicotianamine (Fig. 1) has been proposed (Leete 1964; Sung and Fowden 1971; Leete et al 1974; Kristensen and Larsen

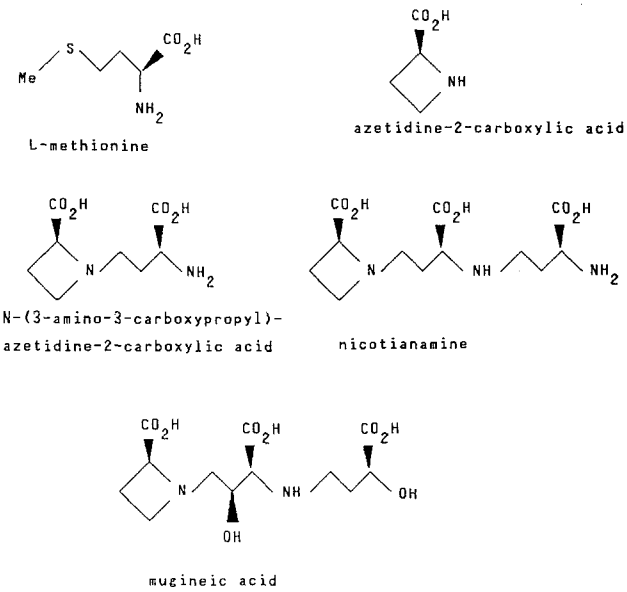

Fig. 1. Chemical structures of mugineic acid and related compounds

Offprint requests to: S. Shojima

1974), this pathway is not based on the direct evidence that L-methionine is incorporated into nicotianamine. If nicotianamine is produced from L-methionine by cultured tobacco cells in the way similar to that by barley roots, these cultured tobacco cells are advantageous for the study of the enzymes involved in the biosynthesis of nicotianamine from L-methionine, because their growth rate is high. Accordingly, we screened seven different kinds of suspension cultures from five species of *Nicotiana* for the occurrence of nicotianamine. Two of the suspension cultures (i.e. *N. megalosiphon* and *N. plumbaginifolia*) contained nicotianamine; further, we found that L-[1-^{14}C]methionine was incorporated into nicotianamine by the suspension culture of *N. megalosiphon* both in vivo and in vitro.

Materials and methods

Seven suspension-cell cultures from five species of *Nicotiana* L. (*N. tabacum* cv. TBY, cv. BY2 and cv. Consolation 402, *N. plumbaginifolia, N. affinis, N. megalosiphon* and *N. africana*) were obtained by the methods reported previously (Nagata et al. 1981; Ishida and Kumashiro 1988). The original habitats of *N. plumbaginifokia, N. affinis, N. megalosiphon* and *N. africana* are South America, South America, Australia and Africa, respectively. The cells were cultured in 95 ml LS medium (pH 5.8) supplemented with KH_2PO_4, 200 mg/l; thiamine·HCl, 1 mg/l; *myo*-inositol, 100 mg/l; sucrose, 3%; and (2,4-dichlorophenoxy)acetic acid, 0.2 mg/l. They were kept in a rotary shaker (90 rpm) at 26.5° C in the dark. One eighth of the suspension-cultured cells were transferred into a fresh medium every week; 15 ml was harvested 7 days after the transfer and then screened.

For the ^{14}C-feeding experiment in vivo, we used the cells of *N. megalosiphon*, in which nicotianamine was detected at the highest concentration. Aliquots of 5 ml were taken from the suspension cell culture at two different growth stages, i.e. at 3 days and 7 days after the renewal of the medium. We refer to them as the 3-day-old cells and the 7-day-old cells, respectively; their fresh mass was 0.3 g and 1.7 g, respectively. L-[1-^{14}C]Methionine (2035 MBq/mmol) with radioactivity of 740 kBq was added to each sample. The samples were incubated for either 3 h or 24 h at 26.5° C in the dark.

Nicotianamine was synthesized in vitro by the method reported previously (Shojima et al. 1989) with some modifications. The minor changes are as follows: 0.2% (mass/vol.) bovine-serum albumin was replaced with 5% (by vol.) glycerol in the homogenizing buffer, and the pH of all the buffers was adjusted to 8.5. The 3-day-old cells of *N. megalosiphon* (11 g fresh mass) were collected on a glass filter and homogenized in 5 ml of the homogenizing buffer with a mortar and pestle. Crude protein fraction was obtained by passing the homogenate through a gel-filtration column and concentrated by ultrafiltration. ATP was added to 4 ml of the concentrated crude protein fraction (1.1 mg protein/ml) at a final concentration of 10 mM. Then, the pH was re-adjusted to 8.5. The reaction was started by adding 740 kBq of L-[1-^{14}C]methionine (2035 MBq/mmol). The samples were incubated at 25° C for 3 h. The experiments were conducted in duplicate. The concentration of the proteins was determined with a Bio-Rad protein assay kit.

The methods for the isolation of the amino acid fraction, analysis by HPLC (an automatic amino acid analyzer) with an on-line radioanalyzer and identification of nicotianamine were described in our previous report (Shojima et al. 1989). The chemical synthesis of nicotianamine and *N*-(3-amino-3-carboxypropyl)azetidine-2-carboxylic acid was reported previously (Fushiya et al. 1981).

Results

Among the cultured cells examined, *N. plumbaginifolia* and *N. megalosiphon* contained nicotianamine at concentrations of 0.03 and 0.10 μmol/g fresh mass, respectively. Because *N. megalosiphon* produced the higher concentration of nicotianamine, L-[1-^{14}C]methionine was incubated with the cultured cells of this species to see if nicotianamine could be produced from L-methionine.

The results obtained on the formation of nicotianamine in vivo after the 24-h incubation of L-[1-^{14}C]methionine with the 7-day-old cells are shown in Fig. 2. Although low radioactivity was detected at the retention time (t_R) of homoserine (t_R 44.4 min) after the 3-h incubation period (data not shown), it disappeared after 24 h of incubation.

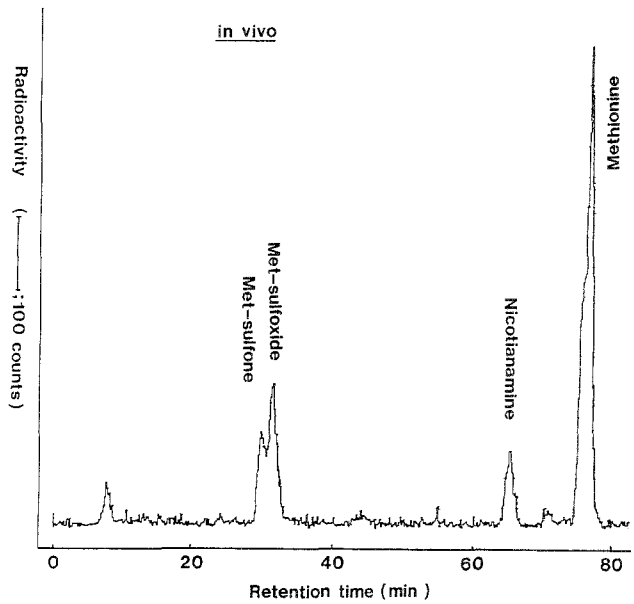

Fig. 2. Incorporation of L-methionine in vivo into nicotianamine by the cultured cells of *N. megalosiphon* after 24 h of incubation. The cells were harvested 7 days after transfer into the fresh medium. From the total volume of amino acid fraction recovered (600 μl), 20 μl was injected into the HPLC at time 0 and detected with the on-line radioanalyzer at a time constant of 105

Table 1. Incorporation of ^{14}C from L-[1-^{14}C]methionine into nicotianamine

Time after inoculation (days)	Incubation time (h)	Radioactivity of NA (10^3 dpm/g fresh mass)	Incorporation of ^{14}C (% total added)
7	3	39	0.15
7	24	110	0.42
3	3	350	0.24
3	24	740	0.50

Nicotianamine (NA) was collected from the HPLC eluate and its radioactivity measured with a liquid scintillation counter. The incorporation of radioactivity into nicotianamine was calculated as a percentage of the total radioactivity of L-[1-^{14}C]methionine added

Under all the experimental conditions, nicotianamine was produced from L-methionine in vivo (Table 1). The ratio of the incorporation of radioactivity into nicotianamine did not differ much between the 3-day-old and 7-day-old cells during the same incubation period. However, the 3-day-old cells showed a greater ability to produce nicotianamine (measured per fresh mass) than the 7-day-old cells (Table 1).

Nicotianamine was also produced in vitro (Fig. 3). Nicotianamine was collected directly from the HPLC eluate and its radioactivity was measured with a liquid-scintillation counter. The activity of the cell-free system to produce nicotianamine was estimated to be about 5800 dpm·mg protein^{-1}·h^{-1}. Assuming that the endogenous L-methionine was absent from the crude protein fraction after the gel filtration, the specific activity was calculated as about 16 pmol nicotianamine·mg protein^{-1}·h^{-1}. Radioactivity was also detected at the retention time of homoserine.

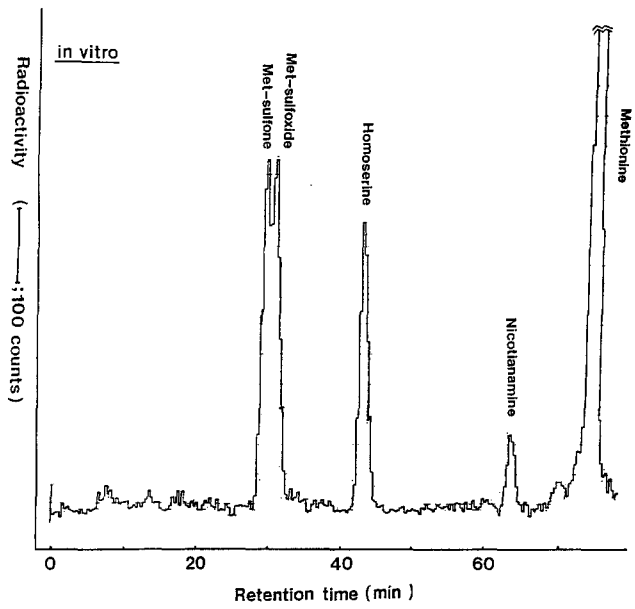

Fig. 3. Incorporation of L-methionine into nicotianamine by the crude protein fraction of *N. megalosiphon* in vitro. From the total volume of amino acid fraction recovered (600 µl), 20 µl was injected into the HPLC at time 0 and detected with the on-line radioanalyzer at a time constant of 20 s

Discussion

The concentration of nicotianamine in the cultured cells of *N. megalosiphon* (0.10 µmol/g fresh mass) and *N. plumbaginifolia* (0.03 µmol/g fresh mass) was as much as those found in tobacco plants (Noma and Noguchi 1976). Since nicotianamine is present widely in the plant kingdom (Budějínský et al. 1980; Rudolph et al. 1985; Schreiber 1985), we had expected that it would be detected in all the cultured cells studied. One reason why the other five suspension cultures did not show nicotianamine may be that the scale of our assay was not large enough (7–10 g fresh mass was sampled from each culture). We think it possible that nicotianamine can be found in the other cultured cells if much more cells are examined. However, the fact that nicotianamine was detected in *N. plumbaginifolia* and *N. megalosiphon* by the small-scale assay indicates that these species are able to produce sufficient nicotianamine for practical isolation of the enzyme involved. In the present study of the biosynthesis of nicotianamine, we used *N. megalosiphon* because it contained nicotianamine at higher concentration than *N. plumbaginifolia*.

Nicotianamine was produced from L-methionine both in vivo and in vitro by the cultured cells of *N. megalosiphon*. Our results show the first direct evidence that L-methionine is a precursor of nicotianamine in *Nicotiana*, as in the case of barley plants (Shojima et al. 1989). However, no peaks of radioactivity were detected at the retention time (t_R) of azetidine-2-carboxylic acid (t_R 40.5 min) or *N*-(3-amino-3-carboxypropyl)azetidine-2-carboxylic acid (t_R 69.4 min). This finding is in good agreement with our earlier proposal that no intermediates are liberated from the enzymes or the enzyme complex during the biosynthesis of nicotianamine (Shojima et al. 1989).

Homoserine seemed to be synthesized by the suspension-cultured cells of *N. megalosiphon* just as in the cell-free system derived from barley roots. Peak 3 in our previous report (Shojima et

al. 1989) proved to be at the same t_R as homoserine. However, there are some differences between the cell-free synthesis by *N. megalosiphon* and that by barley roots. Some unidentified compounds which were produced metabolically in the cell-free system derived from barley roots (Shojima et al. 1989) were not detected in this experiment with tobacco cells. The reason for this difference and whether or not homoserine is the intermediate is presently unknown.

Noma and Noguchi (1976) reported that nicotianamine is present in the highest concentration in the growing leaf tissue of tobacco plants (*N. tabacum*). The difference in the amount of nicotianamine produced per fresh mass (Table 1) may also reflect the difference in the growth rate between the 3-day-old and 7-day-old cells.

Culturing large numbers of tobacco cells is easier than growing the iron-deficient barley plants. The cultured tobacco cells will facilitate the isolation of the enzymes involved in the biosynthesis of nicotianamine from L-methionine. Comparative studies will elucidate the differences in the biosynthetic enzymes produced by tobacco and barley, leading to an understanding of the whole process of the biosynthesis of mugineic-acid-family phytosiderophores in Gramineae.

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