Nicotinic Acid Conjugation in Plants and Plant Cell Cultures of Potato *(Solanum tuberosum)*

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The conversion of nicotinic acid to N-conjugates by either methylation or glucosylation has been investigated in leaves and tubers from potato plants, in potato callus and suspension cultures and in plants regenerated from such cultures. While nicotinic acid-N-glucosyltransferase activity could be detected in the cells and tissues at each level of differentiation, the N-methyltransferase (trigonelline formation) was only found in organized leaf tissue.

Introduction

Nicotinic acid represents an important connecting link between primary and secondary metabolism, because it is a basic element of the coenzymes NAD and NADP and on the other hand it leads into alkaloid metabolism [1]. The ability of nicotinic acid to form conjugates is known for many plant species [2]. In case of heterotrophic cell suspension cultures the rapid alternative conversion of exogenously applied nicotinic acid to either the N-methyl (trigonelline) or the N-glucosyl conjugate has been observed [2, 3]. The enzymes of nicotinate N-conjugation nicotinic acid-N-methyltransferase and nicotinic acid-N-glucosyltransferase have been purified and thoroughly characterized from soybean *(Glycine max)* and parsley *(Petroselinum hortense)* cell suspension cultures, respectively [4, 5].

The formation of nicotinic acid-N-glucoside when measured in heterotrophic suspension cultures seems to be restricted to plant cells of the subclass Asteridae and some higher orders of the subclass Rosidae [3]. However, trigonelline has occasionally been isolated from plants belonging to these highly developed orders [1]. Thus, the possibly alternative mode of conjugation of nicotinic acid in an intact plant and in cell cultures derived thereof needs to be elucidated.

In this report we present data dealing with the formation of the two nicotinate conjugates and the expression of the corresponding enzyme activities, the nicotinic acid-N-methyl and N-glucosyltransferase, in *Solanum tuberosum* tissues and cell cultures. Our studies were performed with cells of different levels of differentiation, but of identical homozygote genetic background. Thus, dihaploid potato plants were compared with cell cultures derived from these plants and with plants regenerated from such cell cultures.

Materials and Methods

Plant material

Potato *(Solanum tuberosum)* tubers of cultivars HH 258 and F 81 tubers were a gift of Prof. Wenzel (Grünbach, F.R.G.). They were used to grow potato plants in a greenhouse. The dihaploid clone HH 258 served as starting material for the cell cultures. F 81 plants had been obtained *via* regeneration of HH 258 protoplasts.

HH 258 callus cultures derived from leaf segments were grown in the dark on solid (0.8% agar) Gamborg B_5 medium [6], pH 5.5, supplemented with 0.2 mg/l 2.4-D, 1 mg/l NAA, 2.5% (v/v) coconut milk and 30 g/l sucrose. HH 258 cell suspension cultures were cultivated in 40 ml of liquid Gamborg B_5 medium without NAA and coconut milk at 25 °C and a weekly subculture.

Callus cultures from which potato plants could be regenerated were cultivated on a modified MS-medium [7] pH 5.8, supplemented with 10 g/l suc-

Abbreviations: 2.4-D, 2.4-dichlorphenoxy acetic acid; NAA, naphtyl acetic acid; IAA, indole acetic acid; 6-BAP, 6-benzylaminopurine; GA3, gibberellic acid; PVP, polyvinylpyrrolidone; UDPG, uridinediphosphoglucose; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.

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rose, 15 g/l mannitol, 1 mg/l IAA, 1 mg/l 6-BAP and 10 mg/l GA_3. A 16 h light period was followed by 8 h of darkness.

Buffer systems

Buffer 1: 0.2 M Tris/HCl, pH 8.0, 14 mM 2-mercaptoethanol. Buffer 2: 0.02 M Tris/HCl, pH 8.0, 5 mM 2-mercaptoethanol. Buffer 3: 0.2 Tris/HCl, pH 8.0, 1 mM dithioerythritol. Buffer 4: 0.02 M Tris/HCl, pH 8.0, 1 mM dithioerythritol. Buffer 5: 0.2 M Tris/HCl, pH 8.0, 1 mM dithioerythritol. Buffer 6: 0.02 M Tris/HCl, pH 8.0, 0.5 M NaCl.

Enzyme preparations

Leaves (2 g fr.wt.) of similar size were thoroughly cleaned and ground in a mortar with 2 g PVP, 2 g quartz sand and 7 ml buffer 1. The mixture was squeezed through three layers of mull cloth. After 30 min of centrifugation at $30{,}000 \times g$ the supernatant was fractionated with solid ammonium sulfate. The protein precipitating between 35–70% saturation was collected by centrifugation and resuspended in 2.5 ml buffer 2. After desalting the solution on a prepacked Sephadex G-25 (PD-10) column (Pharmacia, Uppsala, Sweden) the resulting extract was tested for nicotinic acid-N-methyl and N-glucosyltransferase activities.

Tubers were stored over night at 4 °C in moist paper, peeled and cut into very small pieces. 10 g (fr.wt.) tuber segments were homogenized in a mortar with equal amounts of PVP and quartz sand using 30 ml buffer 3. The following steps were carried out according to procedure A using buffer 4 instead of buffer 2.

Suspension cultured cells (27 g fr.wt.) were harvested 5 days after subculture and ground in a mortar with equal amounts of PVP and quartz sand using 50 ml buffer 5. The homogenate was squeezed through three layers of mull cloth and centrifuged for 30 min at $30{,}000 \times g$. The supernatant was slowly sucked through 12 g PVP previously equilibrated with buffer 3 and packed in a glass funnel. After an ammonium sulfate fractionation according to procedure A the protein pellet was suspended in 2.5 ml buffer 4 and desalted on a Sephadex G-25 (PD-10) column. 2 g DEAE-Sephacel (Pharmacia, Uppsala, Sweden) which had been equilibrated with buffer 4 were then added to the protein solution under careful manual stirring. After 20 min the gel was filtered, washed with buffer 4 to remove unbound protein, suspended in 5 ml buffer 6 and again stirred for 20 min. To collect the bound protein, the gel was filtered and washed with 5 ml buffer 6. The eluted protein was desalted and tested for enzyme activities.

Callus cells (10 g fr.wt.) were extracted according to procedure C with 10 g PVP, 10 g quartz sand and 30 ml buffer 5.

Enzyme assays

The standard assay for UDP-glucose:nicotinic acid-N-glucosyltransferase consisted of 100 µl protein extract, 1.33 mM nicotinic acid containing 3.7 kBq [7-^{14}C]nicotinic acid (The Radiochemical Center Amersham, Amersham, U.K.), 0.5 mM UDPG, 5 mM 2-mercaptoethanol (leaf extracts) or 1 mM dithioerythritol (other extracts) and 0.02 M Tris/HCl pH 8.0 in a total volume of 150 µl. After an incubation period of 2 h at 30 °C the reaction was stopped by transferring the tubes to a boiling water bath.

The enzyme assay for S-adenosylmethionine:nicotinic acid-N-methyltransferase contained 100 µl enzyme preparation, 0.33 mM nicotinic acid with 3.7 kBq [7-^{14}C]nicotinic acid, 0.5 mM S-adenosylmethionine, 27 mM mercaptoethanol (leaf extracts) or 1 mM dithioerythritol (other extracts) and 0.02 M Tris/HCl pH 8.0 in a final volume of 150 µl. The enzyme reaction was allowed to proceed for 2 h at 30 °C and was stopped by transferring the tubes to a boiling water bath.

The suitability of the enzyme assay was checked in mixing experiments with 50 µl of the relevant protein preparation and 50 µl of protein from F 117 potato tubers, which revealed detectable methyltransferase activity.

Product isolation

To separate the enzymatically formed products from the nicotinic acid substrate, the denaturated protein was removed from the assays by centrifugation and then 100 µl of the supernatant were subjected to ion exchange chromatography on Dowex 1X8 (mesh 100–200, formate form). The Dowex-resin (2 ml) had been equilibrated with distilled water and packed into a 5 ml syringe. After application of the enzyme assay solutions trigonelline or nicotinic acid-N-glucoside could be removed by washing the resin with 10 ml distilled water.

Radioactivity in this product fraction was measured by liquid scintillation counting to quantitate the enzyme reactions. Nicotinic acid could subsequently be eluted from the resin with 35 ml 8 M formic acid.

Product identification

The product fractions obtained from ion exchange chromatography were evaporated to dryness and redissolved in 100 µl methanol.

The identity of the enzymatically formed N-methyl and N-glucosyl conjugates was verified using thin layer chromatography on silica gel and cellulose plates (20 × 20 cm, Si GF_{254}, Merck, Darmstadt, F.R.G.) with commercial trigonelline and synthetic nicotinic acid-N-glucoside [8] as references. Chromatographic solvents were 1) *n*-butanol : acetic acid : water (4 : 1 : 1), 2) acetone : water (4 : 1), 3) acetone : water (7 : 3), 4) methanol : chloroform : water (3 : 2 : 1) and 5) *iso*-propanol : water (3 : 1). Radioactivity on the thin layer plates was detected with a scanner (Berthold, Wildbad, F.R.G.).

In addition, the products of the enzymic reactions were subjected to high performance liquid chromatography and the retention time of the enzymatic product was compared with authentic nicotinic acid-N-glucoside and trigonelline.

High performance liquid chromatography

Separation of trigonelline and nicotinic acid-N-glucoside was achieved on a Si 60 LiChrosorb column (250 × 4 mm, 5 µm, Merck, Darmstadt, F.R.G.) monitoring the UV-absorption at 261 nm. Chromatograms were developed using a linear gradient of 100% acetonitrile to 50% acetonitrile in 1.5% (w/v) phosphoric acid within 30 min at a flow of 0.8 ml/min.

Protein determination

The protein content was determined according to Bradford [9] with bovine serum albumine as reference material.

Application of [7-^{14}C]nicotinic acid

The application of [7-^{14}C]nicotinic acid under aseptic conditions followed previous reports [10, 11].

Results

Potato *(Solanum tuberosum)* leaf and tuber tissues, callus cultures and suspension cultured cells were examined for their ability to form N-conjugates of nicotinic acid. The results are depicted in Table I. Leaf extracts of potato plants clone HH 258 were able to convert nicotinic acid to the N-conjugate both by methylation and glucosylation. Nicotinic acid-N-methyl- and N-glucosyltransferase activities could readily be detected. The enzymatically formed products trigonelline and nicotinic acid-N-glucoside were unequivocally identified by TLC and HPLC. Considerable nicotinic acid-N-glucosyltransferase ac-

Table I. Occurrence of nicotinic acid-N-glucoside/nicotinic acid-N-glucosyltransferase and trigonelline/nicotinic acid-N-methyltransferase in plants and cell cultures of *Solanum tuberosum*.

Cell type	Nicotinic acid-N-glucoside		Nicotinic acid-N-glucosyltransferase	Trigonelline		Nicotinic acid-N-methyltransferase
	from enzyme assays	from feeding experiments	(pkat/mg protein)	from enzyme assays	from feeding experiments	(pkat/mg protein)
HH 258 plants						
-leaves	+	n.m.	0.1	+	n.m.	0.3
-tubers	+	n.m.	10.9	−	n.m.	−
HH 258 callus	+	n.m.	45.0	−	n.m.	−
HH 258 suspension cultures	+	+	5.5	−	−	−
F 81 plants (regenerated)						
-leaves	−	+	−	+	+	1.0
-tubers	+	n.m.	2.4	−	n.m.	−

Symbols: +: successful demonstration of conjugate formation.
−: conjugate not formed under experimental conditions used.
n.m.: not measured.

tivity could also be detected in tubers of HH 258 plants, whereas no N-methyltransferase activity could be observed in this material. Using tubers of the tetraploid potato clone F 117 as a control in parallel experiments, methyltransferase activity (0.14 pkat/mg protein) was found and trigonelline could be identified by TLC. The product formation in these enzyme assays was not affected when aliquots of protein from HH 258 tubers were added in mixing experiments. Therefore, the lack of trigonelline formation in extracts from HH 258 tubers is neither due to the procedure of protein preparation nor to the enzyme assay conditions nor to the presence of an inhibitor in the protein preparation.

In heterotrophic callus cells derived from HH 258 plants highest activities of nicotinic acid-N-glucosyltransferase could be measured compared to other potato tissues or cell cultures (Table I), but none of our enzyme preparations from callus cells revealed any methylation capacity for nicotinic acid.

Several experiments with suspension cultured HH 258 cells revealed lower glucosyltransferase activities compared to the callus cultures. However, the enzymatic conversion of nicotinic acid to the N-glucoside could readily be demonstrated, whereas the cells obviously lack the corresponding methyltransferase activity.

The conjugation capacity for nicotinic acid was verified by application of radioactive labelled nicotinic acid to the potato cell suspension cultures. During the linear growth phase 185 kBq [7-^{14}C]nicotinic acid was administered to 4 g cells in the presence of 10^{-4} M non-labelled nicotinate for 24 or 48 h. After separation from the incubation medium, the cells were harvested and subjected to methanolic extraction. Already after 24 h of incubation more than 70% of the applied radioactivity could be extracted from the cells, while less than 0.1% of the nicotinic acid had been converted to $^{14}CO_2$. Cell extracts were subjected to TLC (silica gel plates, solvent systems 3, 1 and 4) to identify the labelled nicotinate conjugates. The major radioactive component in these potato cell extracts was thus shown to be the nicotinic acid-N-glucoside, whereas labelled trigonelline could not be detected in any of the potato cell cultures exposed to radioactive nicotinic acid.

In leaves of the F 81 potato plants, which had been regenerated from HH 258 callus cell protoplasts, nicotinic acid-N-methyltransferase activity was measured with average specific activities of 1.0 pkat/mg protein (Table I), whereas the enzymatic conversion of nicotinic acid to the N-glucoside could not be detected. Application of [7-^{14}C]nicotinic acid for 7 h to leaf disks of such F 81 plants revealed, however, the formation of both the methyl- and the glucosyl conjugates. Nicotinic acid-N-glucoside, trigonelline and free nicotinate were identified by TLC (cellulose plates, solvent systems 1 and 5; silica gel plates, solvent systems 3 and 4). The ratio of N-glucoside to trigonelline was 1:14 estimated by comparing the peak heights on the TLC scanner diagrams. In parallel control experiments with leaves of tetraploid F 117 plants both N-methyltransferase and N-glucosyltransferase activities leading to almost equal amounts of enzymatically synthesized trigonelline and nicotinic acid-N-glucoside could be detected.

These results indicate that in potato plants the occurrence of nicotinic acid-N-methyltransferase activity appears to be linked to the differentiation of leaf tissue. Tubers of F 81 potato plants revealed only nicotinic acid-N-glucosyltransferase but no methyltransferase activity (Table I). The suitability of the N-methyltransferase enzyme assay was confirmed using tubers from F 117 plants and commercially available tubers in control experiments. Mixing experiments with protein from tubers of F 117 and F 81 plants did not indicate the presence of an inhibitor. In the latter cases, enzymatically synthesized trigonelline could clearly be identified by TLC.

Discussion

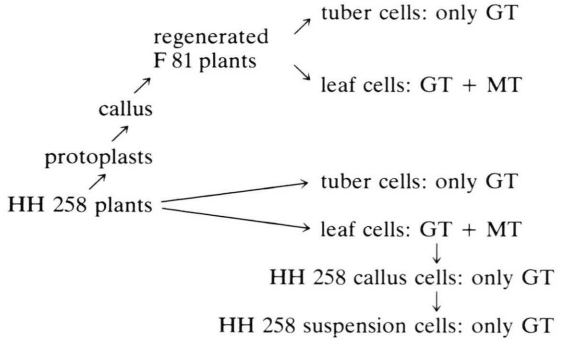
The capacity of potato *(Solanum tuberosum)* cells for the methylation and glucosylation of nicotinic acid was followed through several stages of dedifferentiation from organized plant tissue *via* callus to suspension culture and subsequent regeneration of the plant cormus.

All investigations were aimed at finding glucosyl- and methyltransferase activities rather than quantifying them exactly. In order to detect even small activities of nicotinic acid-N-glucosyl- or N-methyltransferase, the best method of enzyme preparation, large amounts of protein and long incubation periods were used to allow for sufficient conversion of nicotinic acid. As we did not intend to characterize the enzymes neither even to quantify them exactly, a careful check for linearity of the reaction with time or protein in the enzyme assays was not necessary. Therefore the values concerning the specific enzyme

activities are of minor importance; they only indicate the expression of enzyme activity in the various tissues.

It could clearly be demonstrated that the expression of nicotinic acid-N-glucosyl and N-methyltransferase depends on the level of tissue differentiation (Fig. 1). Thus, in cells of callus and suspension cultures, only glucosyltransferase activity could be measured. These cells failed to express enzyme activity for converting nicotinic acid to trigonelline. In contrast, leaf cells of intact potato plants, even when regenerated from callus cells, show both enzyme activities. In tubers, only glucosyltransferase activity could be detected. These results suggest that the synthesis of trigonelline may be regarded as a leaftissue specific process. Tissue and organ specificity has repeatedly been found for many secondary plant metabolites [12]. It had been reported that cell cultures of *Pelargonium* species lack the ability of synthesizing monoterpenes, typical secondary products of the intact plant. After regeneration, however, the plants were able to form monoterpenes with product profiles similar to those of the parent plant [13]. The fact, that in potato plants, the synthesis of trigonelline seems to be restricted to green tissue well corresponds to results obtained with cell cultures of *Nicotiana tabacum,* another Solanaceae plant. In heterotrophic cell suspension cultures of tobacco, only nicotinic acid-N-glucoside could be detected, whereas cells cultivated under photoautotrophic conditions were able to synthesize both the N-glucosyl and the N-methyl conjugate (Ikemeyer and Barz, unpublished). In parsley *(Petroselinum hortense)* cell suspension cultures the N-glucoside has been demonstrated as a storage form of nicotinic acid for pyridine nucleotide synthesis (Upmeier and Barz, unpublished). It is formed as an endogenous constituent and shows rapid turnover with a halflife of 24 h [8]. Synthesis and remobilization is catalyzed by the reversible action of UDP-glucose: nicotinic acid-N-glucosyltransferase [5].

Fig. 1. Expression of Nicotinic acid-N-glucosyltransferase (GT) and nicotinic acid-N-methyltransferase (MT) activity in potato cells of different organization levels.



Studies on nicotinate metabolism in heterotrophic cell cultures of numerous plants [3] revealed, that at the level of suspension cultures only one of the nicotinic acid-N-conjugates is formed by the cells, or at least one of the conjugates is dominating by far. This previous observation has now been substantiated with the potato cell cultures. Though both conjugates are formed in differentiated leaf tissue the N-methyl compound failed to be formed by heterotrophically cultured callus or suspension cells.

The alternative occurrence of nicotinic acid-N-glucoside and trigonelline in cell suspension culture has been considered to be of chemotaxonomic value [14].

However, it is still unclear whether trigonelline may also fulfil the role of a reservoir form for nicotinic acid in suspension cultured plant cells, which are devoid of the N-glucoside. In suspension cultures of soybean *(Glycine max)* trigonelline rather exhibits the properties of a typical secondary plant constituent with low metabolic activity and practically no turnover [8].

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