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Biotransformation of hydroxycoumarins by the cultured cells of *Nicotiana tabacum*

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Abstract

Administration of hydroxycoumarins to the cultured cells of *Nicotiana tabacum* caused the ladder fragmentation of genomic DNA of the cells and the secretion of the intracellular phytoalexin, scopoletin, into the culture medium, and also the exogenous hydroxycoumarins were transformed into their corresponding β-D-glucosides. The glucosylation seems to be a defense reaction of the cells for detoxification of toxic hydroxycoumarins, because their glucosides did not cause the DNA fragmentation.

Keywords: *Nicotiana tabacum*; Cultured cells; Glucosylation; Detoxification; Hydroxycoumarin; Scopoletin; DNA fragmentation; Apoptosis-like cell death

1. Introduction

Plant cell cultures are able to convert exogenous phenolic compounds into their corresponding glucosides [1–9]. It has been reported that glucosylation serves for the detoxification of toxic phenolic compounds which could arise either from normal plant metabolism or from the environment [2,10]. On the other hand, plant cells synthesize phytoalexins in response with defense reaction against phytopathogen and extrinsic chemical stress, which sometimes causes the apoptosis-like cell death [11,12]. The coumarin phytoalexin, scopoletin (6-methoxy-7-hydroxycoumarin, **1**), have been shown to accumulate in some plant tissues in response to fungal and parasitic plant infection, insect attack and mechanical injury [13] and in resistance to disease [14]. In connection with the studies on the practical use of the function of plant cells as biocatalysts, we investigated the defense reaction in the cultured cells of *Nicotiana tabacum* on treatment of hydroxycoumarins as chemical stress, and the metabolic transformation of the hydroxycoumarins by the cells.

2. Experimental

2.1. General experimental procedures

Analytical and preparative TLC was carried out on glass sheets (0.25 mm Merck Silica Gel

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60; GF_{254}). NMR spectra were measured in $CDCl_3$ on a JEOL GSX500 [500 MHz (^1H)] NMR spectrometer. FAB-MS was taken on a JEOL SX102A mass spectrometer. HPLC was carried out with Puresil C_{18} (Waters) column using MeOH:H_2O = 1:3 (v/v) as eluent.

2.2. Cultivation

Suspension cells of *N. tabacum* [15] were cultured in 500 ml conical flasks containing 200 ml Murashige and Skoog's (MS) medium [16] supplemented with 1% sucrose and without auxin (auxin free) under illumination (4000 lux). Cells were cultivated on a rotary shaker (75 rpm) at 25°C for 3 weeks prior to use for biotransformation experiments.

2.3. Preparation and analysis of nuclear DNA

Total DNA was isolated by a slightly modified cetyltrimethylammonium bromide method [17]. The cultured cells of *N. tabacum* were sedimented and freeze-dried in order to remove excess water prior to grinding in liquid N_2. After the isopropanol precipitation step, crude DNA was treated with RNase A, phenol–chloroform extracted and precipitated with ethanol. Isolated DNA was kept in 0.5 ml buffer (10 mM Tris–HCl (pH 7.4) and 1 mM EDTA). Samples (10 μl) and the marker were subjected to electrophoresis on 2% agarose gel in Tris–acetate EDTA buffer. DNA was stained with 0.5 μg ml^{-1} ethidium bromide for 15 min.

2.4. Analysis of scopoletin (*1*) in the cell suspension cultures

To 50 g of the cells portioned to five flasks containing 100 ml of the MS medium, each of 3-hydroxycoumarin (**2**), 4-hydroxycoumarin (**3**), 7-hydroxycoumarin (**4**), and 6,7-dihydroxycoumarin (**5**) (8 mg) was administered and the mixtures were incubated with rotary shaker (75 rpm) at 25°C. At a regular time interval, the cultures were separated from the medium by filtration with nylon mesh and frozen in liquid N_2. Frozen tissues were crashed in a mortar and extracted with Me_2CO–MeOH (1:1). On the other hand, the cultured medium was lyophilized and extracted with Me_2CO–MeOH (1:1). These extracts were separately concentrated under vacuum. Crude scopoletin was subjected to purification with preparative TLC (Merck Silica Gel 60 F_{54}). The TLC plate was developed with benzene–EtOAc–MeOH (1:2:1), and scopoletin was visualized by illuminating the plate with UV light (254 nm). The amount of scopoletin (**1**) was quantified on the basis of the peak area of HPLC on a puresil C_{18} column (150 × 4.6 mm, Waters) eluting at 0.5 ml min^{-1} with MeOH–H_2O (1:3). Scopoletin (**1**) was identified on the basis of spectroscopic analysis: FAB-MS *m/z* 215 $[M + Na]^+$; ^1H NMR (C_5D_5N) δ 6.28 (d, 1H, *J* = 9.4 Hz, H-3), 7.02 (s, 1H, H-5), 7.10 (s, 1H, H-8), 7.66 (d, 1H, *J* = 9.4 Hz, H-4).

2.5. Biotransformation of hydroxycoumarins (*2*–*5*) by the cultured cells of *N. tabacum*

Five milligrams of the substrates such as (**2**–**4**) and **5** dissolved in 50 μl EtOH was administered at 1-day intervals to the flask containing the suspension cells (150 g) of *N. tabacum* in 500 ml of MS medium. A total of 0.1 mmol of the substrate (15 mg) was administered. The cultures were incubated at 25°C on a rotary shaker (75 rpm) for an additional 5 days after final administration. After incubation, the cells and medium were separated by filtration. The cells were extracted with MeOH and the extract was concentrated by evaporation in vacuo. The methanolic fraction was partitioned between H_2O and *n*-BuOH. The filtered medium was extracted with *n*-BuOH. These butanolic fractions were subjected to preparative TLC with MeOH:$CHCl_3$ (1:4) to give 0.7 mg of 3-*O*-β-D-glucopyranosylcoumarin (**6**), 0.8 mg of 4-*O*-β-D-glucopyranosylcoumarin (**7**), 3 mg of 7-*O*-β-D-glucopyranosylcoumarin (**8**), 6 mg of 6-*O*-β-D-glucopyranosyl-7-hydroxycoumarin

(**9**). The structures of the glucosides were identified on the basis of ^{1}H NMR and FAB-MS.

6: FAB-MS *m/z* 325 $[M+H]^+$; ^{1}H NMR (C_5D_5N) δ 4.14–4.38 (m, 5H, H-2′,3′,4′,5′,6b′), 4.58 (dd, 1H, $J = 12.2$, 1.8 Hz, H-6a′), 5.72 (d, 1H, $J = 7.6$ Hz, H-1′), 7.08 (td, 1H, $J = 8.5$, 1.2 Hz, H-6), 7.26 (td, 1H, $J = 8.5$, 1.2 Hz, H-7), 7.33 (dd, 1H, $J = 7.9$, 1.5 Hz, H-5), 7.53 (dd, 1H, $J = 7.9$, 1.5 Hz, H-8), 7.61 (s, 1H, H-4).

7: FAB-MS *m/z* 325 $[M+H]^+$; ^{1}H NMR (C_5D_5N) δ 4.37–4.41 (m, 5H, H-2′,3′,4′,5′,6b′), 4.48 (dd, 1H, $J = 12.2$, 1.5 Hz, H-6a′), 5.86 (d, 1H, $J = 6.1$ Hz, H-1′), 6.47 (s, 1H, H-3), 7.05 (td, 1H, $J = 8.2$, 1.5 Hz, H-6), 7.24 (dd, 1H, $J = 8.2$, 1.5 Hz, H-6), 7.42 (td, 1H, $J = 8.2$, 1.5 Hz, H-7), 7.78 (dd, 1H, $J = 8.2$, 1.5 Hz, H-8).

8: FAB-MS *m/z* 325 $[M+H]^+$; ^{1}H NMR (C_5D_5N) δ 4.14–4.40 (m, 5H, H-2′,3′,4′,5′,6b′), 4.54 (dd, 1H, $J = 12.2$, 1.5 Hz, H-6a′), 5.70 (d, 1H, $J = 7.6$ Hz, H-1′), 6.28 (d, 1H, $J = 9.4$ Hz, H-3), 7.15 (dd, 1H, $J = 8.5$, 2.4 Hz, H-6), 7.23 (d, 1H, $J = 2.4$ Hz, H-8), 7.33 (d, 1H, $J = 8.5$ Hz, H-5), 7.59 (d, 1H, $J = 9.4$ Hz, H-4).

9: FAB-MS *m/z* 363 $[M+Na]^+$; ^{1}H NMR (C_5D_5N) δ 4.06–4.40 (m, 5H, H-2′,3′,4′,5′,6b′), 4.54 (dd, 1H, $J = 12.2$, 2.1 Hz, H-6a′), 5.58 (d, 1H, $J = 7.9$ Hz, H-1′), 6.21 (d, 1H, $J = 9.4$ Hz, H-3), 7.08 (s, 1H, H-8), 7.52 (d, 1H, $J = 9.4$ Hz, H-4), 7.62 (s, 1H, H-5).

*2.6. Time course experiments in the glucosylation of hydroxycoumarins (**2–5**)*

The suspension cells of *N. tabacum* were cultured for 2–3 weeks. Each 50 g of the cells was portioned to seven flasks containing 100 ml of the MS medium. Hydroxycoumarins (**2–5**) (8 mg) were administered to each of the flasks, and the mixtures were incubated with rotary shaker (75 rpm) at 25°C. At a regular time interval, one of the cultures was extracted with MeOH, and then the methanolic fraction was partitioned between H_2O and EtOAc. The aqueous layer was further extracted with *n*-BuOH. The filtered medium was extracted with EtOAc and then with *n*-BuOH. The amounts of the glucosides in the butanolic fractions were calculated on the basis of the peak area of their HPLC analyses.

3. Results and discussion

3.1. Effects of hydroxycoumarins on nuclear DNA of the cultured cells of N. tabacum

To clarify the effect of the hydroxycoumarins on the cultured cells of *N. tabacum*, each of (**2–4**) and **5** was fed to the suspension cultures of *N. tabacum*. After 3 days incubation, it was observed that the cells with the administration of **4** or **5** turned brown, but, in contrast, the cells with **2** or **3** remained healthy. Therefore, after 2 days incubation with **4**, DNA was examined by the agarose gel electrophoresis. As shown in Fig. 1, the genomic DNA was found to be fragmented in a ladder form. This indicates that an apoptosis-like cell death [12] may be induced by administration of **4** in the cultured cells of *N. tabacum*. **5** also induced the DNA fragmentation, but **2** and **3** did not. In contrast to the case of **4**, no DNA fragmentation was observed in the cultured cells treated with the corresponding glucoside (**8**). These results suggest that the coumarins with phenolic hydroxyl group cause

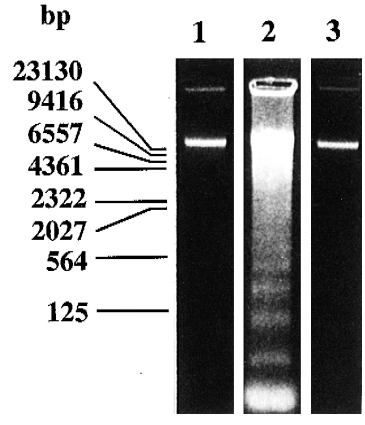

Fig. 1. Agarose gel electrophoresis of DNA extracts from the cultured cells of *N. tabacum*. The cells were treated with the following coumarins: lane 1, none; lane 2, **4**; lane 3, **8**.

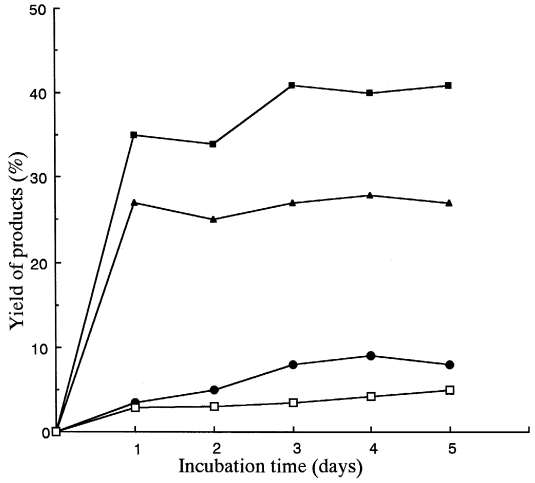

Fig. 2. Time courses in the glucosylation of **2** (– • –), **3** (–□–), **4** (–▲–) and **5** (–■–) by the cultured cells of *N. tabacum*.

the DNA ladder fragmentation characteristic of the apoptosis-like death of the cells.

3.2. Glucosylation of hydroxycoumarins with the cultured cells of N. tabacum

Administration of (**2–4**) to the cell suspension cultures yielded their corresponding *O*-β-D-glucopyranosylcoumarins (**6–8**), respectively. In the case of **5**, the position of glucosylation was specific to 6-hydroxy group, yielding **9**. The rate of glucosylation of the hydroxycoumarins depended on the position of hydroxyl group, e.g., the cultured cells glucosylated rapidly **4** rather than **2** and **3** by a factor of 4, as shown in Fig. 2. **5** was more rapidly converted to the corresponding monoglucoside (**9**). These observations indicate that the cultured cells of *N. tabacum* have capability to glucosylate exogenous coumarins having harmful phenolic hydroxyl group.

3.3. Accumulation and secretion of scopoletin (1) by feeding of hydroxycoumarins to the cells

To clarify the secretion of the phytoalexin, scopoletin (**1**) in the cell suspension cultures, time courses of the scopoletin content in the cultured cells of *N. tabacum* on feeding the hydroxycoumarins were followed. The content of scopoletin (**1**) in the cultured cells (Fig. 3A) and the culture medium (Fig. 3B) was found to increase gradually by the administration of **4** and **5**, whereas the increase of the scopoletin content was not found in the case of the administration of **2** and **3**. On the other hand, when **8** was administered to the cells, no secretion of scopoletin (**1**) in the cultured medium even after 5 days incubation. These results indicate that the cultured cells of *N. tabacum* transform the hydroxycoumarins, **4** and **5**, to the intracellular phytoalexin, scopoletin (**1**), and then secrete it into the cultured.

Thus, it was clarified that administration of harmful hydroxycoumarins to the cultured cells of *N. tabacum* caused the transformation of the hydroxycoumarins into their corresponding β-

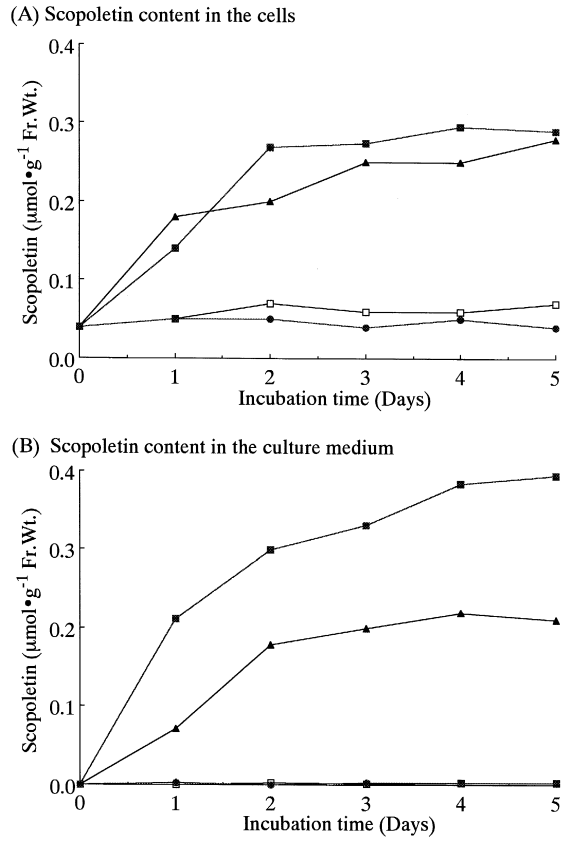

Fig. 3. Time courses of the scopoletin (**1**) content in the cultures of *N. tabacum* after administration of hydroxycoumarins: (– • –) **2**, (–□–) **3**, (–▲–) **4**, (–■–) **5**. (A) Scopoletin content in the cells. (B) Scopoletin content in the culture medium.

D-glucosides and also the formation of the phytoalexin, scopoletin (**1**). The glucosylation seems to be a kind of defense reaction of the cells for detoxification of toxic hydroxycoumarins, because their glucosides did not cause the fragmentation of genomic DNA.

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