Production of Tropane Alkaloids during De-differentiation of *Scopolia parviflora* Calli

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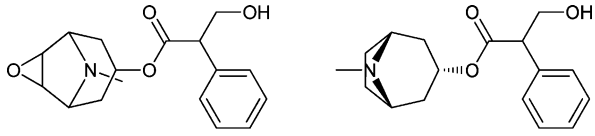
The production of tropane alkaloids during differentiation and de-differentiation of *Scopolia parviflora* calli was studied. Tropane alkaloid production drastically decreased during calli de-differentiation. Scopolamine (**1**) production decreased after 10 days of culture, whereas that of hyoscyamine (**2**) decreased during de-differentiation of root to calli. The production of **1** was enhanced in calli undergoing differentiation to shoot after 60 days of culture, reaching a maximum by 80 days. However, production of hyoscyamine in regenerated plants was lower. The expression level of hyoscyamine 6β-hydroxylase (H6H), a key biosynthetic enzyme for tropane alkaloids, was significantly increased in 4-week-old calli. This study suggests that the biosynthesis of tropane alkaloids is regulated inversely in de-differentiating *Scopolia parviflora* calli.

Secondary metabolism is closely linked to plant differentiation processes.[1] Secondary metabolite biogenesis is generally restricted to a particular tissue and a specific developmental stage, whereas accumulation may occur throughout the plant.[2] Some morphologically specialized plant tissues or organs that elaborate medicinal compounds have been produced in culture systems by induction of specific organ cultures or by adopting undifferentiated cell cultures.[3] Cultured cells and organ tissues show fluctuations in the level and nature of secondary metabolites depending upon culture periods. Generally, low metabolite levels are observed in these cultures due to lack of cell differentiation.[4] Incidences reporting the overproduction of secondary metabolites by adopting tissue cultures are on the rise, whereas the studies concerning the production of secondary metabolites during a particular developmental stage are less understood.

The tropane alkaloids scopolamine (**1**) and hyoscyamine (**2**) are among the earliest drugs used in traditional medicine.[5] These alkaloids are mainly synthesized in the roots and later translocated to aerial plant parts.[6] Studies on the production of tropane alkaloids by cultured cells and root tissues of various members of the *Solanaceae*[7–9] have revealed the importance of differentiated root tissue for the production of alkaloids. Furthermore, reports on the production of tropane alkaloids during differentiation and de-differentiation stages of *Scopolia parviflora* and the reports on the enzymes involved therein are scanty. This study is therefore directed toward gathering experimental data on the developmental influences on biosynthesis of tropane alkaloids in *S. parviflora*.



1 **2**

Results and Discussion

Ten day-old adventitious roots were used to generate calli (Figure 1A). The root surfaces developed calli after 20 days of culture on B5 solid medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (Figure 1B). Calli further developed and increased in size until 30 days; thereafter, their propagation was rapid (Figure 1C). After 40 days friable calli and hairy roots were obtained (Figure 1D).

The production of alkaloids scopolamine and hyoscyamine (**2**) during de-differentiation of adventitious roots to calli were monitored by HPLC analysis (Figure 2). The concentration alkaloids, particularly of scopolamine, decreased to 0.7 mg/g DW (dry weight) during de-differentiation in the first 10 days of culturing. After 20 days of culture, scopolamine levels in the adventitious roots decreased further and remained stable until day 40. On the other hand, the levels of hyoscyamine during de-differentiation were lower than scopolamine on a comparative basis.

The selected organogenic calli derived from adventitious roots (Figure 1D) were subcultured on B5 medium supplemented with 3% sucrose and 2,4-D (4.52 μM) at 4-week intervals for organogenesis (Figure 3B). The color of the organogenic calli turned to yellow-green within 2 weeks on cytokinin medium (Figure 3C) and turned to green within 3 weeks (Figure 3D). After 4 weeks of culture, small primodia appeared within organogenic calli. Organized cell clumps were seen in 40-day-old organogenic calli that produced shoot buds. Calli lines that were cultured on B5 medium supplemented with BA (benzylaminopurine) gave rise to primordia within 4 weeks (Figure 3E), which further developed into to entire shoots (Figure 3F).

The production of tropane alkaloids by organogenic and non-organogenic *S. parviflora* calli was determined (Figure 4). The organogenic calli cultured on B5 medium supplemented with BA (22.2 μM) for 10 days contained 0.7 mg/g DW of scopolamine and 0.17 mg/g DW of hyoscyamine. After 60 days, the scopolamine content of the calli began to increase dramatically to reach a maximal level in 80 days (organogenesis stage). However the scopolamine levels thereafter decreased rapidly in regenerated plants. The levels of hyoscyamine during differentiation stages were generally low at the corresponding culture periods (Figure 4), and the level was lower than that of scopolamine.

H6H is a bifunctional enzyme encoded by the H6H gene. H6H hydroxylates hyoscyamine to 6β-hydroxyhyoscyamine and also mediates epoxidation of scopolamine. The expression of H6H in

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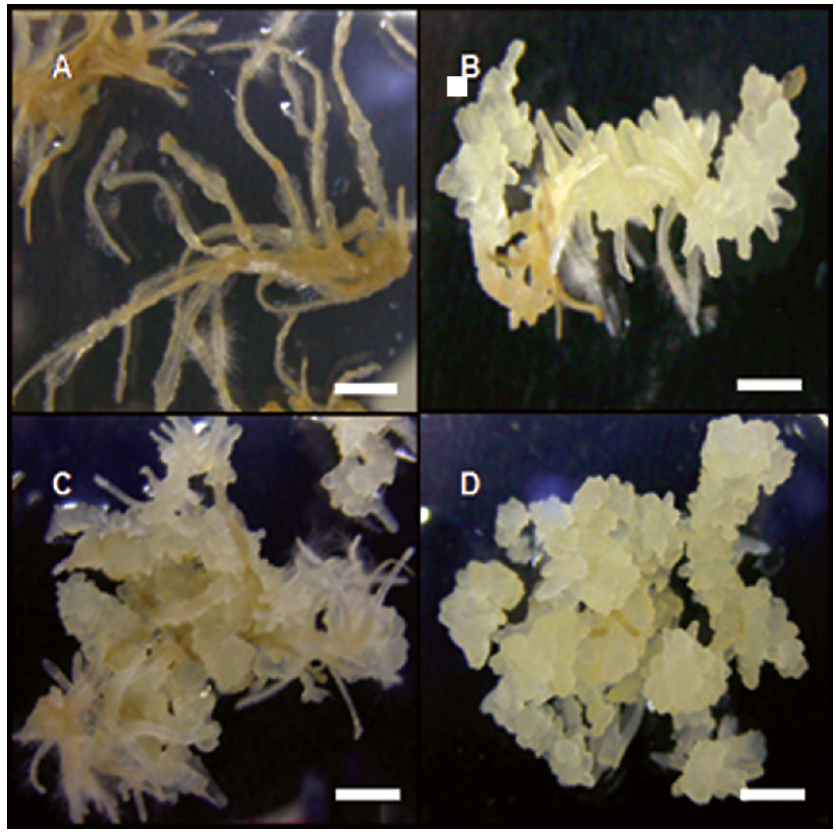

Figure 1. De-differentiation of adventitious root to calli on B5 solid medium supplemented with 3% sucrose and various levels of 2,4-D. (A) Adventitious root (bar = 1.0 cm). (B) Calli formation after 20 days of culture (bar = 1.5 cm). (C) Further growth of induced calli after 30 days of culture (bar = 2.3 cm). (D) Friable calli after 40 days of culture (bar = 2.5 cm).

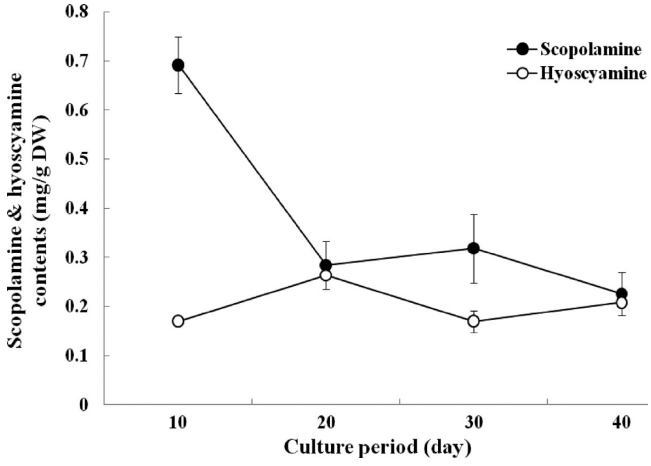

Figure 2. Tropane alkaloid content of adventitious roots at the de-differentiation stage. The adventitious roots were cultured on B5 medium supplemented with 2.26 μM 2,4-D for de-differentiation from root to callus, and alkaloids were determined by HPLC.

adventitious roots, nonorganogenic calli, and organogenic calli was examined by western blot analysis (Figure 5).

The expressional analysis showed abundant H6H-specific bands in the lanes corresponding to the extracts obtained from adventitious roots and faint bands in the extracts derived from nonorganogenic calli. The adventitious root H6H protein profile was similar to 2-week-old organogenic calli. However, H6H-specific protein flux was higher in extracts starting from 4 weeks onward.

The extent of tropane alkaloids biosynthesis decreased during the de-differentiation procedure. Especially, the scopolamine levels decreased drastically after 10 days of culture. The alkaloids are known to be biosynthesized initially in the root cells and then translocated to other plant parts for storage.[6] Khanam et al.[10] reported that scopolamine and hyoscyamine were absent in the 2-week-old green calli; however, they were detected in the 11-week-old nonorganogenic calli of *Duboisia myoporoides.* We reported earlier that nonorganogenic calli produced small amounts of tropane alkaloids.[10] Similar observations were recorded in *Catharanthus roseous*.[11] In calli induced from the seedlings, vinblastine yield decreased sharply and the calli produced 1.6 μg/g DW. However our results suggest that alkaloid biosynthesis is negatively regulated during de-differentiation of *S. parviflora*.

Organogenic calli regenerate in 90 days starting from the initial calli induction stage. Alkaloid analyses of the organogenic calli indicated a sharp increase in scopolamine that depended on culture periods, reaching a maximal level in 80 days. Thus, the biosynthesis of alkaloids, and probably other secondary metabolites, is related to cell differentiation stages. Datta and Srivastava[11] reported that vinblastine could be detected in calli lines established from different explants. As calli differentiated into multiple shoots, vinblastine production by them also increased rapidly, equalizing to that of in vivo seedlings of similar age. The high degree of differentiation and maturity in the tissues of *Catharanthus* was correlated to the increased vinblastine production, both in vivo and in vitro. Kitamura et al.[12] also reported that leaves of the *D. myoporoides* seedlings contained the major alkaloids throughout development, whereas leaves of plants regenerated from calli contained few if any alkaloids.

Scopolamine content in de-differentiated tissues was high compared to that of hyoscyamine. As in an earlier study of Kim et al.,[13] the scoplolamine content in regenerated plants or organogenic calli was high compared to hyoscyamine. This result supposes that scopolamine biosynthesis is influenced by the cell differentiation process. Although the cell differentiation process in *S. parviflora*

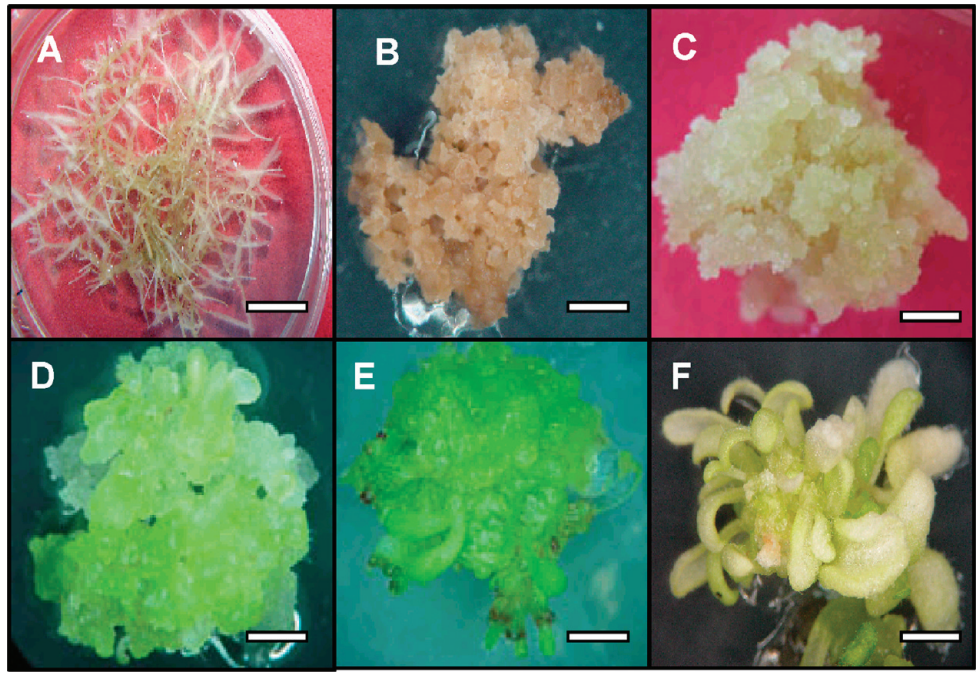

Figure 3. De-differentiation of adventitious hairy roots to calli on B5 medium supplemented with 0.45 μM 2,4-D (A−C) and differentiation of organogenic calli on B5 medium supplemented with 8.88 μM BA (D−F). (A) Adventitious root (bar = 1.0 cm). (B) Nonorganogenic calli (bar = 2.3 cm). (C) Organogenic calli (bar = 2.5 cm). (D) Primodia induced from the organogenic calli after 60 days of culture (bar = 2.7 cm). (E) Induction of secondary primordia after 80 days of culture (bar = 3.1 cm). (F) Numerous shoot regeneration from primordia after 90 days (bar = 3.6 cm).

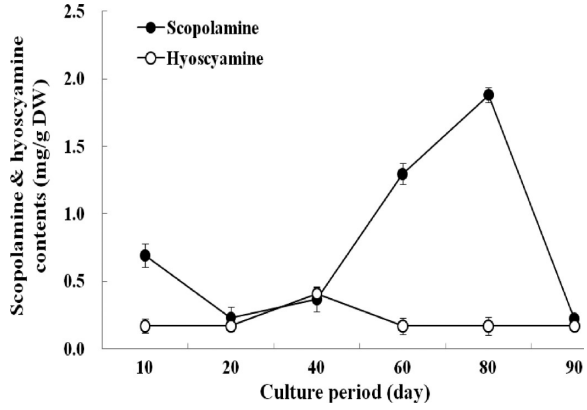

Figure 4. Tropane alkaloid production over time (during various stages of differentiation). Organogenic calli were cultured on B5 solid medium supplemented with 8.88 μM BA and alkaloids determined by HPLC.

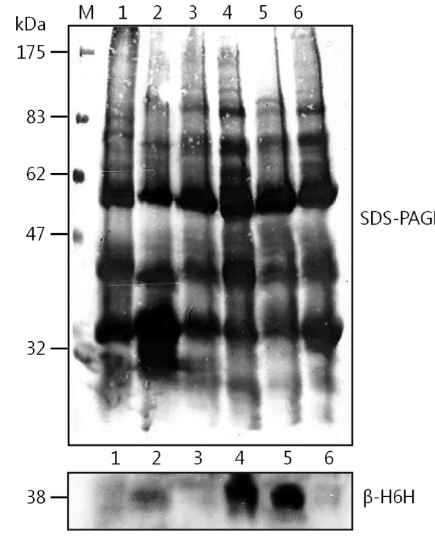

Figure 5. SDS-PAGE patterns (top panel) and western blot analysis (bottom panel) of H6H. Lane M, size marker. Lane 1, adventitious root. Lane 2, nonorganogenic calli. Lane 3, organogenic calli (40-day culture). Lane 4, organogenic calli (60-day culture). Lane 5, organogenic calli (80-day culture). Lane 6, regenerated plant (90-day culture).

led to improvements in alkaloid yields, these quantities were only comparable to alkaloid production by hairy root cultures and hairy roots. Nevertheless, this study shows that the cell differentiation process can contribute to development of efficient in vitro methods for boosting alkaloid production.

The expression level of H6H was consistent with tissue sources that correspond to the highest production of tropane alkaloids. It was of interest to note that expression of the H6H gene positively correlated with culture periods in organogenic calli, and the same was negatively regulated in nonorganogenic calli. This study thus implies that cell differentiation influences the expression of H6H enzyme. Also the pattern of cellular H6H gene expression correlated with production of scopolamine. Organogenesis may affect scopolamine accumulation through both an increase of the alkaloid precursor pool and an enhancement of several enzymes involved in the biosynthesis of tropane alkaloids. This is inconsistent with the fact that the regulation of the biosynthetic pathway of secondary metabolites is modulated by different developmental and physiological mechanisms operating at a particular growth phase.[11]

In conclusion, this study suggests that the differentiation stages of *S. parviflora* are important for the biosynthesis of tropane alkaloids, especially of scopolamine. The de-differentiated tissues have a limited capacity to biosynthesize such compounds. However, further work is required to elucidate roles of other enzymes and genes related to secondary metabolite biosynthesis during differentiation and de-differentiation stages of plants.

Experimental Section

Plant Material. *Scopolia parviflora,* used in this experiment, was collected from Korea National Arboretum, Korea. The plant was taxonomically identified and verified by Dr. C. H. Lee (Division of Plant Conservation, Korea National Arboretum), and voucher specimens were deposited. The adventitious roots of *S. parviflora* were induced from the rhizome of mature plants as reported.[14] The organogenic and nonorganogenic calli were selected from adventitious root-derived calli.[13]

Extraction and Determination of Tropane Alkloids. Tropane alkaloids from plant material were determined by HPLC according to Kang et al.[16]

De-differentiation of Adventitious Roots. The root and in vitro adventitious root segments (about 10 mm length without root tip) were cultured on B5[15] solid medium supplemented with 3% sucrose and various levels of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.45, 2.26, 4.52, and 9.05 μM). Calli induced from roots were subcultured on B5 medium containing 3% sucrose and 2,4-D (2.26 μM) at 4-week intervals. The cultures were incubated at 25 ± 2 °C in the dark.

Differentiation from Organogenic Calli. Actively growing organogenic calli (0.5 g fresh wt) were transferred to 9 mm Petri dishs containing B5 solid medium supplemented with BA (8.88 μM). Organogenic calli from the regeneration medium were picked and selected using a light microscope (Olympus, Japan) and cultured on B5 medium with 2,4-D (0.45 μM) for 4 weeks. All cultures were incubated adopting a 16/8 h (light/dark) photoperiod at 25 ± 2 °C for 8 weeks. The cultures were maintained by subculturing to fresh medium at 4-week intervals.

Hyoscyamine 6β-Hydroxylase (H6H) Expressional Analysis. Expression levels of H6H during alkaloid biosynthesis in *S. parviflora* were investigated by conducting western blotting.[17] Plant materials at different differentiation stages were collected, frozen in liquid nitrogen, and homogenized. The cell homogenates were suspended in a protein extraction buffer (300 mM Tris-HCl (pH 8.0), 1 mM EDTA) and centrifuged at 12 000 rpm for 15 min. The protein concentration of the supernatants was measured using the Biorad protein kit. Crude cell extracts (20 μg/lane) were separated by SDS-PAGE on a 12.5% separating gel according to Laemmli.[18]

Immuno-detection of H6H was carried out using the anti-H6H polyclonal antibody as the primary antibody (gift from Dr. Hashimoto of NAIST, Japan). Unbound antibody was removed with three washes using TTBS (500 mM Tris-HCl (pH 7.4), 1.5 M NaCl, and 1% Tween 20). The antibody bound to the nitrocellulose membrane was detected using peroxidase-conjugated antimouse IgG as the secondary antibody. The antigen protein was detected employing an ECL kit following the protocol provided by the manufacturer.

Statistical Analysis. Data are expressed as an average of three separate experiments. The bars indicate standard deviation from the mean of each replicate treatment.

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