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Biosynthesis of Brassinolide from Teasterone via Typhasterol and Castasterone in Cultured Cells of *Catharanthus roseus**

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Abstract. The biosynthesis of brassinolide (BL) in crown gall and nontransformed cells of *Catharanthus roseus* in which BL, castasterone (CS), typhasterol (TY), and teasterone (TE) are endogenous was investigated using deuterated TY and TE as substrates. The metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS) and/or GC-selected ion monitoring (SIM). It was found that these cells converted TY to CS and BL, as well as TE to TY and CS. Because the pathway from CS to BL in the cells has already been confirmed, a biosynthetic sequence of TE → TY → CS → BL was established. Reversible conversion between TE and TY was observed.

Crown gall cell line, V208 (nopaline-type) of *Catharanthus roseus* produces castasterone (CS) and brassinolide (BL) (Park et al. 1989), their contents being comparable to those of such brassinosteroid-rich plant tissues as pollens and immature seeds. Recently, octopine-type cells (V277) and nontransformed cells (Vn) of *C. roseus* have also been confirmed to produce CS and BL (Choi et al. 1993, Suzuki et al. 1993). It has been found that CS was convertible into BL by feeding [^{3}H]CS to V208 cells (Yokota et al. 1990) and a mixture of [^{2}H]CS and [^{3}H]CS to V208, V277, and Vn cells (Suzuki et al. 1993). Thus CS has been recognized as a biosynthetic precursor of BL in nontransformed and transformed cells. Campesterol or its analogs are expected to be converted to teasterone (TE) through several oxidative steps, then successively to typhasterol (TY), CS, and BL (Yokota et al. 1991). However, no evidence has been obtained for the hypothesized pathway except the final step.

This article reports the occurrence of TE and TY in the nontransformed and transformed cells of *C. roseus* as well as the biosynthesis of BL in the cells using deuterium-labeled TE and TY as substrates.

Materials and Methods

Cell Lines and Culture Conditions

Three cell lines of *C. roseus*, crown gall cells of nopaline type, crown gall cells of octopine-type, and nontransformed cells were cultured as described in the previous article (Suzuki et al. 1993).

Labeled Substrates

[26,28-$^2H_6$]TE, [26,28-$^2H_6$]TY, [26,28-$^2H_6$]CS, and [26,28-$^2H_6$]BL have been synthesized as already described (Takatsuto and Ikekawa 1986). $^2H_6$ contents were determined to be more than 99% by mass spectrometry.

Bioassay

Rice lamina inclination test was carried out using a cultivar Koshihikari (Arima et al. 1984).

Extraction of Cells for the Identification of Endogenous Brassinosteroids

In the first experiment, 2 kg fresh weight (fr wt) of V208 cells grown for 14 days (stationary phase) were collected and immediately extracted with MeOH using a homogenizer. The MeOH

* Biosynthesis of brassinosteroids in *Catharanthus roseus*. Part III. Part II of this series: Suzuki et al. (1993).

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was evaporated in vacuo to give an aqueous solution, which was adjusted to pH 3 and extracted with EtOAc. The organic phase was washed with saturated $NaHCO_3$, dried over anhydrous Na_2SO_4, and concentrated to give a neutral EtOAc-soluble (NE) fraction. In the second experiment, V208 cells (200 g fr wt), V277 cells (180 g fr wt), and Vn cells (200 g fr wt) were collected after an 8-day culture (mid log phase), extracted with MeOH, and mixed with internal standards ($[^2H_6]$TY and $[^2H_6]$TE; 0.5 ng/g fr wt each) prior to solvent fractionation.

Silica Gel Adsorption Chromatography

The NE fraction in the first experiment was put onto a column packed with 25 g of silica gel (Wakogel C-300). The column was eluted stepwise with 250 ml each of $CHCl_3$, $CHCl_3$-MeOH (97:3, 95:5, 93:7, 90:10, 80:20) and MeOH. The NE fraction from the second experiment was charged onto a column packed with 5 g of silica gel. The column was eluted with 50 ml each of $CHCl_3$ and $CHCl_3$-MeOH (93:7).

Sephadex LH-20 Chromatography

Sephadex LH-20 chromatography (column volume; 200 ml) was conducted using a solvent system of MeOH-$CHCl_3$ (4:1).

High Performance Liquid Chromatography (HPLC)

A large-scale HPLC was conducted with a Senshu Pak ODS column (10 × 50 mm + 20 × 250 mm), eluted with 65% aqueous MeCN at a flow rate of 8 ml/min. A small-scale HPLC was conducted on an ODS column (10 × 150 mm), eluted with 75% aqueous MeCN at a flow rate of 2 ml/min.

Feeding Experiments

The deuterated compounds in 75% EtOH (5 μg/10 μl) were added to a 200-ml conical flask in which cells (6–15 g fr wt) had been grown for certain periods in 60 ml of a medium as described above and incubated for 24 or 48 h at 27°C in the dark on a reciprocal shaker (100 rpm).

Extraction and Purification of the Metabolites

The cells and medium were mixed with MeOH, and homogenized. After evaporation of the solvent, the resulting aqueous solution was extracted with $CHCl_3$. The $CHCl_3$-soluble fraction was purified with silica gel and C_{18} cartridge columns according to the methods described in our previous article (Suzuki et al. 1993). Final purification was carried out by HPLC on a Senshu Pak ODS column (10 × 50 mm + 20 × 250 mm) under the same conditions described above to yield BL fraction [retention time (Rt) 9–12 min], CS fraction (Rt 12–16 min), TE fraction (Rt 20–26 min), and TY fraction (Rt 26–31 min).

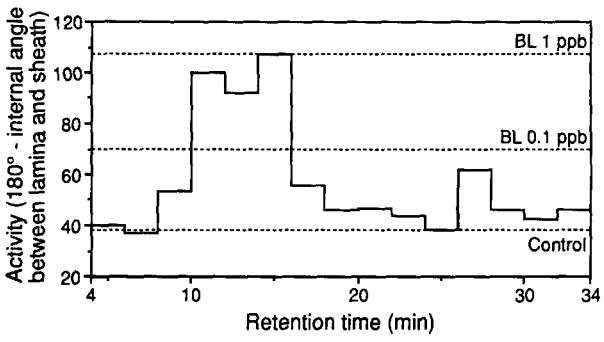

Fig. 1. Distribution of biological activity after ODS-HPLC of the extract from *Catharanthus roseus* crown gall cells (V208). Biological activity was determined by the rice lamina inclination test.

Gas Chromatography-Mass Spectrometry (GC-MS) and GC-Selected Ion Monitoring (SIM) Analysis

The CS and BL fractions were treated with 10 μl of pyridine containing 20 μg of methaneboronic acid at 70°C for 30 min. The TE and TY fractions were treated with methaneboronic acid as described above, and then with 10 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide at 70°C for 30 min. The analyses were carried out on a Finnigan MAT INCOS 50 mass spectrometer connected with a Hewlett-Packard 5890A gas chromatograph as described in our previous article (Suzuki et al. 1993). Because of the slight difference in the column length, Rts of the authentic compounds in each experiment varied. In an experiment, Rts of the authentic compounds were as follows: $[^2H_6]$TE, 11.32 min; $[^2H_6]$TY, 10.82 min; $[^2H_6]$CS, 11.20 min; $[^2H_6]$BL, 11.77 min.

Results

Identification of TE and TY in Cultured Cells of Catharanthus roseus

The NE fraction from 14-day-old V208 cells (stationary phase) was purified on silica gel, yielding biologically active fractions eluted with 3–7% of MeOH in $CHCl_3$. Further purification on Sephadex LH-20 afforded a biologically active fraction (Ve/Vt: 0.6–0.8), which was purified by large-scale HPLC to give three biologically active fractions with Rts corresponding to BL (10–12 min), CS (12–16 min), and TY (26–28 min) as shown in Fig. 1. No significant biological activity was found in the TE fraction (Rt 20–24 min). The TY fraction was derivatized to methaneboronate-trimethylsilyl ether and subjected to GC-SIM analysis. Ions characteristic of the derivatized TY were observed at m/z 544 $[M^+]$, 529, 515, and 454, and the Rt was the same as the authentic specimen. Thus TY was found to be endogenous in V208 cells.

The second experiment to examine the occur-

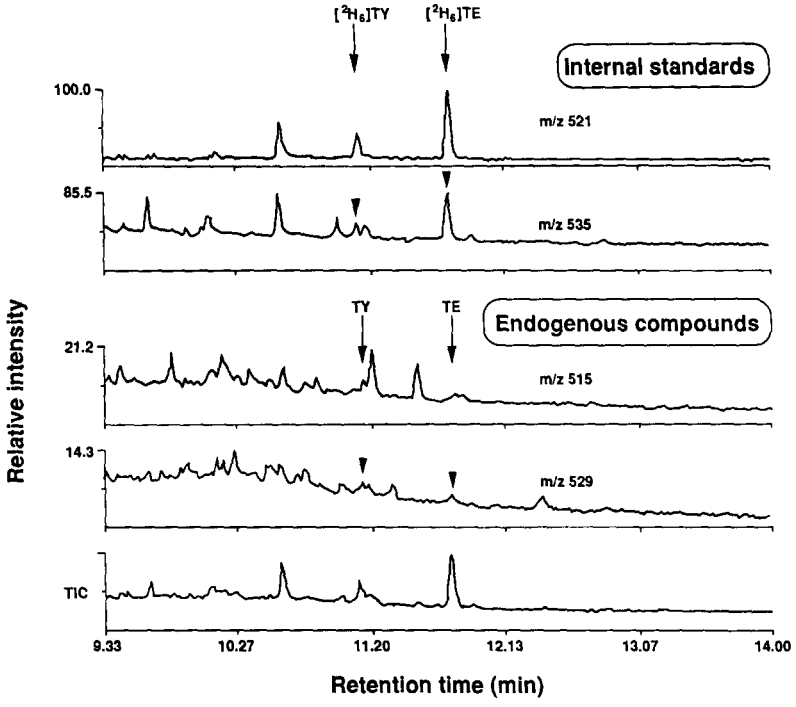

Fig. 2. GC-SIM identification of TY and TE from partially purified extract of *Catharanthus roseus* nontransformed cells (Vn). [$^2H_6$]TY and [$^2H_6$]TE were added to the extract prior to purification.

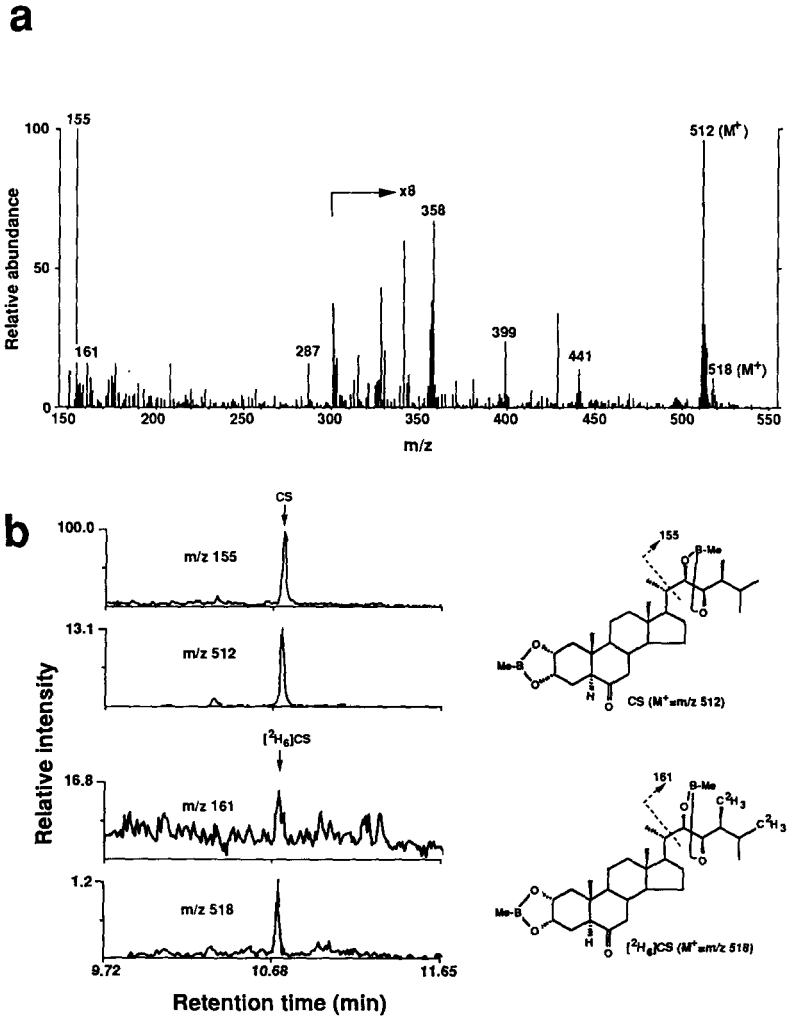

Fig. 3. GC-MS identification of [$^2H_6$]CS converted from [$^2H_6$]TY in *Catharanthus roseus* crown gall cells (V208). (**a**) Mass spectrum. (**b**) Mass chromatogram.

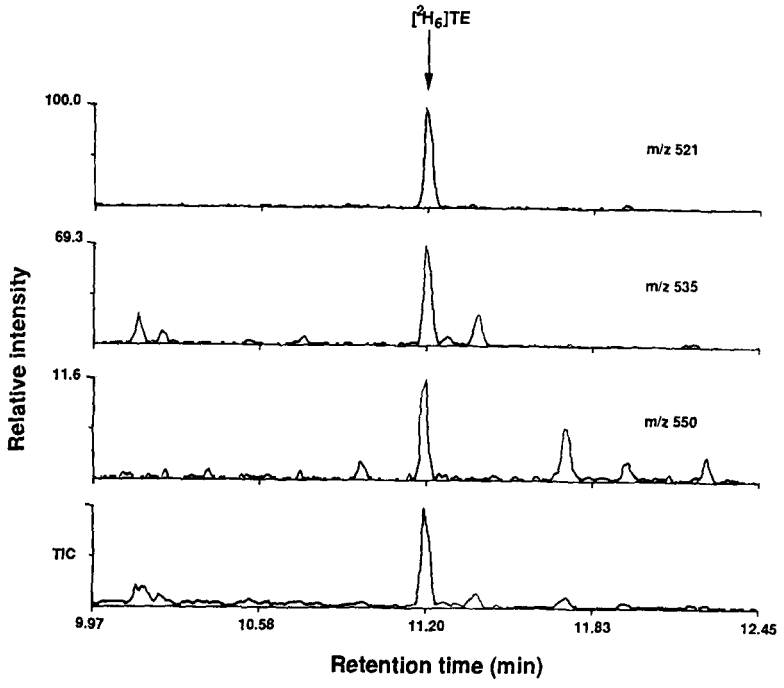

Fig. 4. GC-SIM identification of $[^2H_6]$TE converted from $[^2H_6]$TY in *Catharanthus roseus* crown gall cells (V208).

rence of TY and TE in V208, V277, and Vn cells of *C. roseus* was carried out using 8-day-old cells (mid log phase). $[^2H_6]$TY and $[^2H_6]$TE were added as internal standards to the MeOH extract. The NE fraction was purified on silica gel. The eluate with 7% MeOH in $CHCl_3$ was purified on Sephadex LH-20 as described above. Purification by large-scale HPLC (Rt 20–33 min) followed by small-scale HPLC (Rt 8–14 min) afforded a combined fraction of TE and TY which was analyzed by GC-SIM. For the derivatives of TY and TE, ions at m/z 529 $[M^+-15]$ and 515 $[M^+-29]$ were monitored, and for the corresponding deuterated derivatives, ions at m/z 535 $[M^+-15]$ and 521 $[M^+-29]$ were monitored. In the case of Vn cells (Fig. 2), small ion peaks due to endogenous TY (Rt 11.15 min) and TE (Rt 11.76 min) were observed along with the ion peaks due to internal standards ($[^2H_6]$TY at 11.13 min and $[^2H_6]$TE at 11.73 min). Thus, TY and TE were identified from Vn cells. Similarly, TY and TE were identified from V208 and V277 cells by GC/SIM. Contents of TE and TY in the three cell lines were much lower than endogenous levels of CS and BL (1–5 ng/g fr wt) (Yokota et al. 1990) and roughly estimated to be less than 50 pg/g fr wt.

The Metabolism of TY

Three cell lines in the late log phase (~9–10 days old) were incubated with $[^2H_6]$TY (V208, for 24 or 48 h; V277 and Vn, for 48 h). As described in Materials and Methods, metabolites were separated into TE, TY, CS, and BL fractions.

Table 1. Incorporation of $[^2H_6]$TY into $[^2H_6]$TE, $[^2H_6]$CS, and $[^2H_6]$BL in *Catharanthus roseus* cultured cells

	Incorporation rate (%) into brassinosteroid after 48 h incubation		
	$[^2H_6]$TE	$[^2H_6]$CS	$[^2H_6]$BL
Cell lines			
V208	0.1	0.3	ND[a]
V277	0.1	0.3	0.3
Vn	0.1	0.5	ND

Note: Incorporation rate was calculated from GC-MS or -SIM data and expressed as percentage to $[^2H_6]$TY (5 μg) fed to the cells.
ND, Not detected.
[a] In the metabolites of 24 h incubation, $[^2H_6]$BL was detected with incorporation rate of 0.1%.

The CS fraction obtained from V208 cells was analyzed by GC-MS after conversion to the bismethaneboronate derivative. In the mass spectrum obtained from the metabolites of 48 h incubation (Fig. 3a), ions due to $[^2H_6]$CS were clearly observed at m/z 518 $[M^+]$ and 161, although ions due to endogenous CS were prominent. In the mass chromatogram (Fig. 3b), ions due to $[^2H_6]$CS (a metabolite of $[^2H_6]$TY) and endogenous CS were separately observed at 10.70 and 10.72 min, respectively. Similar result was obtained from the metabolites of 24 h incubation. In GC-SIM analysis

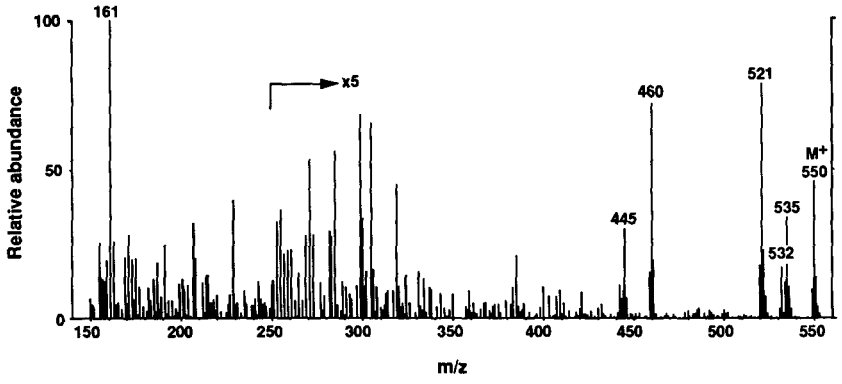

Fig. 5. Mass spectrum of [$^2H_6$]TY converted from [$^2H_6$]TE in *Catharanthus roseus* crown gall cells (V208).

of the BL fraction from the metabolites of 24 h incubation, ions due to [$^2H_6$]BL bismethaneboronate (m/z 534 [M^+], 374, 338, 177, and 161) were clearly detected at the same Rt (11.22 min) as the authentic specimen. From the metabolites of 48 h incubation, [$^2H_6$]BL was not observed.

Similarly, in V277 cells, conversion of [$^2H_6$]TY to [$^2H_6$]CS and [$^2H_6$]BL was clearly demonstrated by GC-SIM. In Vn cells, only conversion of [$^2H_6$]TY to [$^2H_6$]CS was observed. Unexpectedly, [$^2H_6$]TE was also identified from TE fractions of the three cell lines (Fig. 4).

Thus, it was found that metabolites of TY were CS, BL, and TE in *C. roseus* cells. Conversion rates of [$^2H_6$]TY to [$^2H_6$]CS, [$^2H_6$]BL, and [$^2H_6$]TE were roughly estimated from the heights of ion peaks observed in GC-MS or -SIM analyses (Table 1).

The Metabolism of TE

V208, V277, and Vn cells in the late log phase (~9–10 days old) were incubated with [$^2H_6$]TE for 48 h. As in the case of the [$^2H_6$]TY feeding, metabolites were separated into TE, TY, CS, and BL fractions.

The TY fractions obtained from the three cell lines were analyzed by GC-MS. In all cases Rt (10.82 min) and mass spectrum were identical to [$^2H_6$]TY. Fig. 5 shows the spectrum obtained from V208 cells. The CS fractions obtained from the three cells lines were analyzed by GC-SIM with monitoring ions at m/z 518 [M^+], 358, 287, and 161 characteristic of bismethaneboronate of [$^2H_6$]CS. In all cases, SIM profiles identical to authentic specimen were obtained. Thus TE was found to be convertible to TY and CS in V208, V277, and Vn cells. The conversion rates were obtained by GC-MS or -SIM analysis (Table 2). The incorporation rate of [$^2H_6$]TE to [$^2H_6$]TY exceeded 35% in cases of V208 and V277 cells, whereas incorporation to [$^2H_6$]CS was much lower.

Table 2. Incorporation of [$^2H_6$]TE into [$^2H_6$]TY and [$^2H_6$]CS in *Catharanthus roseus* cultured cells

	Incorporation rate (%) into brassinosteroid after 48 h incubation	
	[$^2H_6$]TY	[$^2H_6$]CS
Cell lines		
V208	63.9	2.7
V277	35.5	0.8
Vn	5.7	0.2

Note. Incorporation rate was calculated from GC-MS or -SIM data and expressed as percentage to [$^2H_6$]TE (5 μg) fed to the cells.

Discussion

This study revealed the occurrence of TE and TY in addition to the major BRs, CS and BL, in *C. roseus* crown gall cells and nontransformed cells. These BRs are all included in the hypothetical pathway of BL biosynthesis.

Although a small difference was observed between transformed and nontransformed cells, it seems that essentially identical biosynthetic pathways of BL are present in these cells. The above cell lines all converted TE to TY and CS. Effective conversion of TE to TY suggests that TE should be a direct precursor of TY. This pathway was found to be reversible, although the reverse reaction of TY to TE was not predominant (Tables 1 and 2). TY was converted to both CS and BL. In earlier works, we demonstrated that CS was a precursor of BL in these cells (Suzuki et al. 1993, Yokota et al. 1990). therefore, it was established that TE was converted to BL via TY and CS (Fig. 6).

The reversible conversion between TE and TY predominantly preferred the formation of 3α-hydroxyl of axial configuration (TY) to the formation of 3β-hydroxyl of equatorial configuration (TE) (Tables 1 and 2). The mechanism of the reaction is

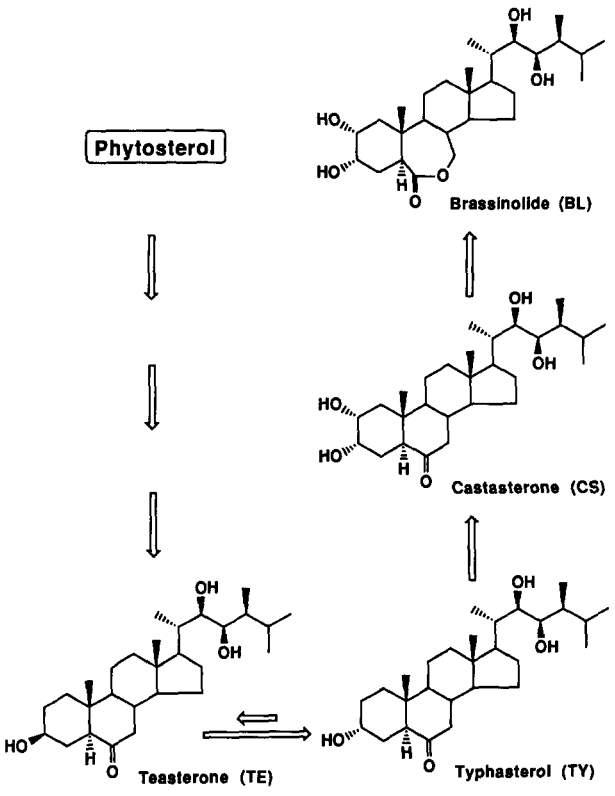

Fig. 6. Biosynthetic pathway of BL in nontransformed and transformed cells of *Catharanthus roseus*.

quite of interest from a chemical point of view. In this reversible inversion, either of the two following enzymatic reactions might be possible, namely, 1) one-step inversion of 3β(α)-hydroxyl to 3α(β)-hydroxyl or, 2) oxidation of 3β(α)-hydroxyl to 3-one and subsequent reduction to 3α(β)-hydroxyl. To find whether the 3-oxo compound is present in the metabolites will be quite important when clarifying the inversion mechanism of the 3-hydroxyl.

Since the conversion of TE to BL via TY and CS was found in both nontransformed cells (Vn) and crown gall cells (V208 and V277), the enzymes responsible for BL biosynthesis appears to be independent of T-DNA genes. So far, co-occurrence of TE, TY, CS, and BL has been demonstrated in five plant species, *Phaseolus vulgaris* (Kim 1991), *Citrus unshiu* (Abe 1991), *Thea sinensis* (Morishita et al. 1983, Abe et al. 1984), *Lilium elegans* (Suzuki et al. unpublished results), and *Catharanthus roseus* (present study). Therefore, it seems that the biosynthetic pathway of BL found in this study might be ubiquitous in higher plants.

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References

Abe H (1991) Rice-lamina inclination, endogenous levels in plant tissues and accumulation during pollen development of brassinosteroids. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity, and applications, American Chemical Society Symposium series no. 474. American Chemical Society, Washington, DC, pp 200–207

Abe H, Morishita T, Uchiyama M, Takatsuto S, Ikekawa N (1984) A new brassinolide-related steroid in the leaves of *Thea sinensis*. Agric Biol Chem 48:2171–2172

Arima M, Yokota T, Takahashi N (1984) Identification and quantification of brassinolide-related steroids in the insect gall and healthy tissue of the chestnut plant. Phytochemistry 23:1587–1591

Choi YH, Inoue T, Fujioka S, Saimoto H, Sakurai A (1993) Identification of brassinosteroid-like active substances in plant-cell cultures. Biosci Biotech Biochem 57:860–861

Kim SK (1991) Natural occurrence of brassinosteroids. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity, and applications, American Chemical Society Symposium series no. 474. American Chemical Society, Washington, DC, pp 26–35

Morishita T, Abe H, Uchiyama M, Marumo S, Takatsuto S, Ikekawa N (1983) Evidence for plant growth promoting brassinosteroids in leaves of *Thea sinensis*. Phytochemistry 22:1051–1053

Park KH, Saimoto H, Nakagawa S, Sakurai A, Yokota T, Takahashi N, Syōno K (1989) Occurrence of brassinolide and castasterone in crown gall cells of *Catharanthus roseus*. Agric Biol Chem 53:805–811

Suzuki H, Fujioka S, Takatsuto S, Yokota T, Murofushi N, Sakurai A (1993) Biosynthesis of brassinolide from castasterone in cultured cells of *Catharanthus roseus*. J Plant Growth Regul 12:101–106

Takatsuto S, Ikekawa N (1986) Synthesis of deuterio-labelled brassinosteroids, [26, 28-$^2H_6$]brassinolide, [26, 28-$^2H_6$]castasterone, [26, 28-$^2H_6$]typhasterol and [26, 28-$^2H_6$]teasterone. Chem Pharm Bull 34:4045–4049

Yokota T, Ogino Y, Suzuki H, Takahashi N, Saimoto H, Fujioka S, Sakurai A (1991) Metabolism and biosynthesis of brassinosteroids. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity, and applications, American Chemical Society Symposium series no. 474. American Chemical Society, Washington, DC, pp 86–96

Yokota T, Ogino Y, Takahashi N, Saimoto H, Fujioka S, Sakurai A (1990) Brassinolide is biosynthesized from castasterone in *Catharanthus roseus* crown gall cells. Agric Biol Chem 54:1107–1108