Identification and Transformation of Campestanol in Cultured Cells of *Phaseolus vulgaris*

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Brassinosteroids (BRs) are a new family of steroidal plant hormones that regulate plant growth and differentiation.[1-4] To date, more than forty naturally-occurring BRs have been identified in the entire plant kingdom.[5-6] Among them, brassinolide (BL, see structure in Figure 1) and its direct biosynthetic precursor, castasterone (CS), have been considered as the most important BRs because of their strong biological activity and wide-distribution in plant kingdom. Recent feeding experiments using isotope-labeled substrates and molecular genetic analyses of BR-related mutants have revealed that both BRs are biosynthesized from campesteol (CR), a phytosterol that has the same carbon skeleton as those of the BRs, via two biosynthetic pathways, namely the early and late C6-oxidation pathway.[7-10]

It has been demonstrated that *Phaseolus vulgaris* contain teasterone (TE), typhasterol (TY), 6-deoxocastasterone (6-deoxoCS), CS and BL.[5] These BRs are members of the early or/and late C6-oxidation pathway to produce BL, indicating that both biosynthetic pathways are functional in the plant. In both pathways, CR is initially converted into campestanol (CN), and CN is then converted into 6-oxoCN *via* 6α-hydroxyCN and 6-deoxocathasterone (6-deoxoCT) in the early and late C6-oxidation pathway, respectively.[8,10,11] Therefore, CN is an important biosynthetic intermediate from which two pathways are split and whose conversion to 6-oxoCN or 6-deoxoCT may reflect which pathway is dominantly operative in the early stage of BRs biosynthesis in a plant where both pathways are found. Nevertheless, the presence and conversion of CN in *P. vulgaris* have not been investigated yet. To get further information on the biosynthesis of BRs in *P. vulgaris*, we examined the presence and enzymatic transformation of CN in *Phaseolus* cells.

The unsaponified lipid obtained from *n*-hexane soluble fraction of *Phaseolus* cells was subjected to a silica gel column eluted with a mixture of *n*-hexane and methylene chloride. Based on the movement on a F_{254} preparative TLC, 4-demethylsterols were separated from non-polar lipids, 4,4-dimethylsterols and 4-methylsterols. After the 4-demethylsterols were acetylated, the obtained 4-demethylsteryl acetates were analyzed by a capillary GC-MS. As summarized in Table 1, a peak at 17.38 min on total ion chromatogram gave prominent ions at *m/z* 444 (M^+, relative intensity 20), 384 (39), 369 (30), 276 (34), 257 (9) and 215 (100) whose retention time on GC and mass spectrum were identical to those of authentic campestanyl acetate, indicating that the active principle in the peak was campestanyl acetate. Therefore, the presence of CN was demonstrated in the *Phaseolus* cells.

Studies have shown that enzymes catalyzing the conversions from CN to 6-oxoCN *via* 6α-hydroxyCN and 6-deoxoCT, namely CN-6α-hydroxylase, 6α-hydroxyCN reductase and 22*R*-hydroxylase, respectively, are cytochrome P450 proteins which are bound to membrane of cells, most likely endoplasmic reticulum (E.R.).[12,13] Thus, a microsomal enzyme solution to test the conversions was prepared from cultured cells of *P. vulgaris* by ultra-centrifugation. The obtained microsomal solution showed a strong activity for NADPH cytochrome c reductase (an E.R. marker)[14] but no alcohol dehyrogenase (a cytosolic marker),[15] indicating that microsomal enzymes were suitably prepared without contamination of cytosolic enzymes.

First, enzymatic conversion from CN to 6-oxoCN *via* 6α-hydroxyCN in *Phaseolus* cells was examined. To that end, deuterium-labeled CN ([$^2H_6$]-CN) and NADPH as a substrate and a cofactor, respectively, were added to the microsomal enzyme solution. After incubation, deuterium-unlabeled ($^2H_0$) expected products, [$^2H_0$]-6-oxoCN and [$^2H_0$]-6α-hydroxyCN, were added to the assay mixture for quantitative analyses. The mixture was then purified by methods described in Experimental section, and the enzyme products were analyzed by a capillary GC-selected ion monitoring (SIM) after trimethylsilylation.

In GC-SIM analysis, diTMSi ether of an enzyme product gave selected ions at *m/z* 568 (M^+) and 553 (M^+-15) to detect [$^2H_6$]-6α-hydroxyCN-diTMSi ether at the same retention time (17.4 min) as that of authentic [$^2H_6$]-6α-hydroxyCN-diTMSi ether (Figure 2A). Thus, the product was characterized to be [$^2H_6$]-6α-hydroxyCN. MonoTMSi ether of another product exhibited characteristic ions to determine [$^2H_6$]-6-oxoCN-TMSi ether at *m/z* 494 (M^+), 479 (M^+-15) and 465 (M^+-29) at a retention time identical to that of authentic [$^2H_6$]-6-oxoCN-TMSi (Figure 2B), indicating that the product was [$^2H_6$]-6-oxoCN. In consequence, [$^2H_6$]-6α-hydroxyCN and [$^2H_6$]-6-oxoCN were successfully identified as the enzyme products of [$^2H_6$]-CN, by which the presence for two enzymes catalyzing the C6-oxidation, CN 6α-hydroxylase and 6α-hydroxyCN reductase in the *Phaseolus* cells was demonstrated. The specific enzyme activity measured

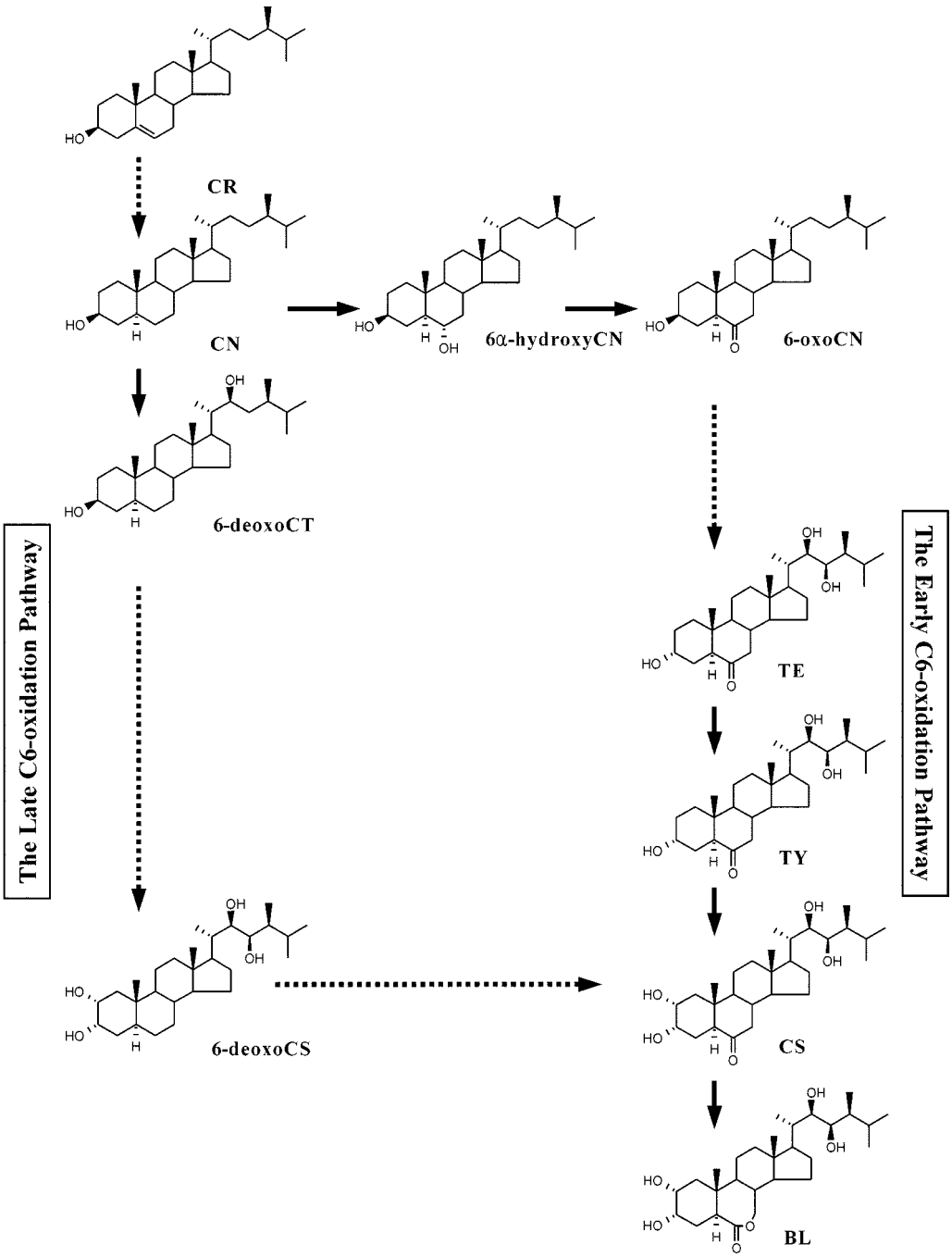

Figure 1. BRs biosynthetic pathway confirmed in *P. vulgaris*. Solid and dotted arrow indicates a single- and mutiple-reaction, respectively.

by ratio of [$^2H_6$]-product/[$^2H_0$]-internal standard in the assay mixture for CN 6α-hydroxylase and 6α-hydroxyCN reductase was 14.0 and 13.9 ng mg protein^{-1}min^{-1}, respectively.

Next, enzymatic conversion of CN to 6-deoxoCT in *Phaseolus* cells was assayed by the same methods described above except addition of [$^2H_0$]-6-deoxoCT as an internal standard for quantitative analysis. Because diTMSi ether of [$^2H_6$]-6-deoxoCT gave no strong characteristic ions except for the base peak ion at *m/z* 193 in mass spectrum, determination of the product was monitored by the base peak at

Table 1. Identification of CN from *P. vulgaris* by GC-MS

Compound[a]	Rt[b] on GC	Prominent ions (*m/z*, relative intensity %)
Endogenous CN	17.38	444 (M^+, 20), 384 (39), 369 (30), 276 (34), 257 (9), 215 (100)
Authentic CN	17.38	444 (M^+, 18), 384 (38), 369 (32), 276 (35), 257 (11), 215 (100)

[a]Sample was analyzed as an acetate derivative. [b]Rt: Retention time

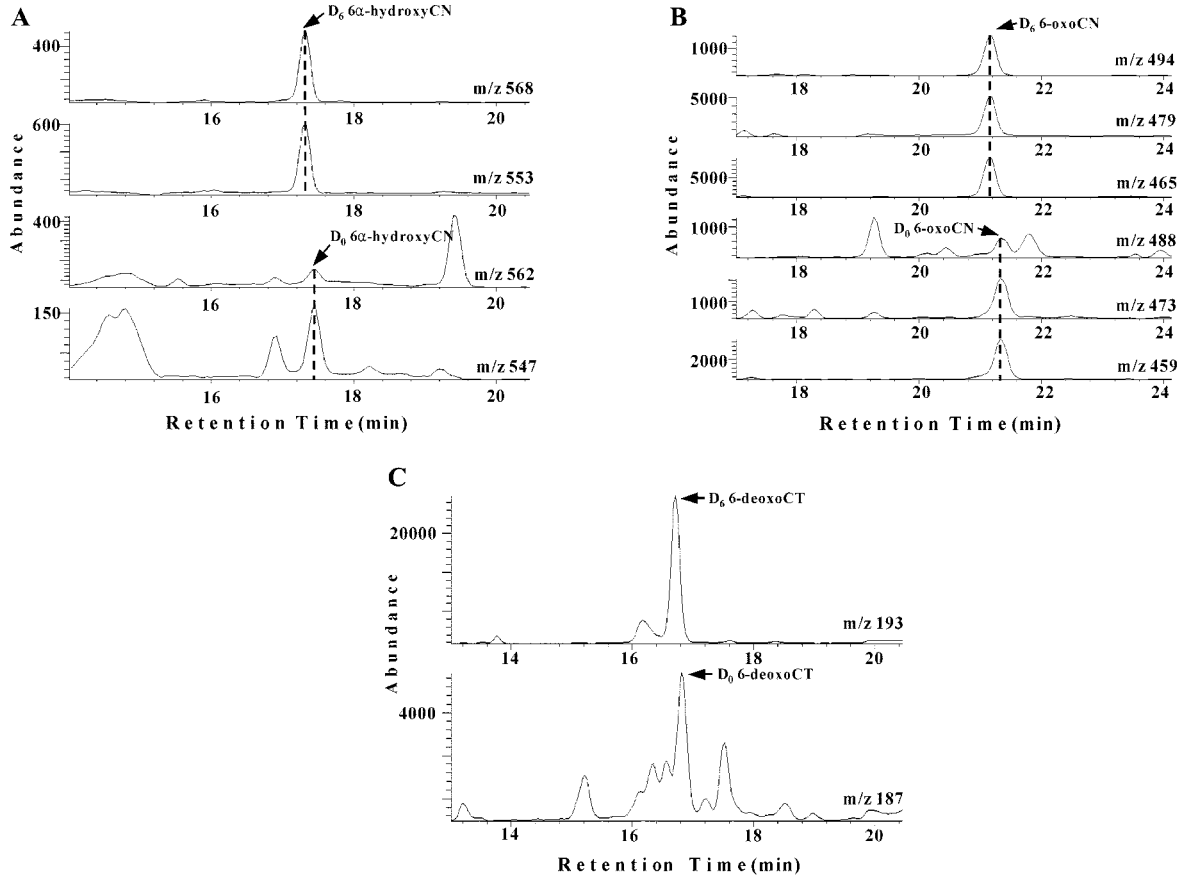

Figure 2. GC-SIM analysis of enzyme products for CN hydroxylase (A), 6α-hydroxyCN reductase (B) and CN 22*R*-hydroxylase (C) in *P. vulgaris* cells.

m/z 193. As shown in Figure 2C, ion at *m/z* 193 showed the same retention time as that of authentic [$^2H_6$]-6-deoxoCT-diTMSi, demonstrating that the product was [$^2H_6$]-6-deoxoCT. Therefore, conversion of [$^2H_6$]-CN to [$^2H_6$]-6-deoxoCT by CN 22*R*-hydroxylase in *Phaseolus* cells was demonstrated. The specific activity for CN 22*R*-hydroxylase in the cells was calculated to be 5.1 ng mg protein^{-1}min^{-1} based on ratio of [$^2H_6$]-product/[$^2H_0$]-internal standard.

The present study is the first to show the presence of CN in *Phaeolus vulgaris* cell. In addition, the presence of three BRs biosynthetic enzymes catalyzing conversion of CN to 6-oxoCN *via* 6α-hydroxyCN and to 6-deoxoCT in the cells is demonstrated for the first time. Together with the occurrence of CR and endogenous BRs, such as TE, TY, 6-deoxoCS, CS and BL, in *Phaseolus* cells,[16-19] these results indicate both that the early and late C6-oxidation are involved in synthesis of endogenous BRs, and CN is a common biosynthetic precursor for both biosynthetic pathways in the cells. The specific activity of CN 6α-hydroxylase and 6α-hydroxyCN reductase for the conversion from CN to 6-oxoCN *via* 6α-hydroxyCN in the early C6-oxidation pathway was approximately 2.7 times higher than that of CN 22*R*-hydroxylase for the conversion from CN to 6-deoxoCT in the late C6-oxidation pathway in the *Phaseolus* cells, indicating that two thirds and one third of CN is converted into 6-oxoCN and 6-deoxoCN in the cells, respectively. This strongly suggests that the early C6-oxidation pathway is predominantly operative compared with the late C6-oxidation pathway in the early stage of BRs biosynthesis in *Phaseolus* cells.

Experimental Section

Identification of CN in *Phaseolus vulgaris*. Suspension

Table 2. Enzyme activities in the microsomal fraction obtained from *P. vulgaris* cells

Subcellular fraction	Enzyme				
	NADPH cytochrome c reductase[a]	Alcohol dehydrogenase[a]	CN hydroxylase[b]	6α-OHCN reductase[b]	CN 22*R*-hydroxylase[b]
Microsomal	1.9	N.D	14.0	13.9	5.1

[a]The specific activity of enzyme was expressed as μmol of the product mg protein^{-1} min^{-1}. [b]The specific activity of enzyme was expressed as ng of the product mg protein^{-1}min^{-1}. N.D: Not Detected

cultured cells of *P. vulgaris* (500 g) were homogenized with 80% methanol (1.5 L), and extracted with *n*-hexane (1.5 L). The dried residue of *n*-hexane soluble fraction (0.5 g) was saponified with 80% ethanol (200 mL) containing 5% KOH at 70 °C for 90 min. The unsaponified lipids (400 mg) were extracted with *n*-hexane and charged on silica gel column (2.2 × 200 mm) using a mixture (1 : 1) of *n*-hexane and methylene chloride as an elution solvent. Based on a F_{254} TLC (developing solvent: ethanol free chloroform), the 4-demethylsterol fraction (410 mg) was obtained. The 4-demethylsterols were dissolved in acetic anhydride (2 mL) and pyridine (4 mL), and incubated at room temperature for 18 h. The products were then adjusted to pH 7 with HCl solution (pH 3), and extracted with *n*-hexane. After the removal of *n*-hexane, 4-demethylsteryl acetates were collected and subjected to GC-MS.

Enzyme preparation and assay. The cultured cells (10 g) were ground in a mortar and pestle at 4 °C in 20 mL of 0.2 M Tris-HCl (pH 7.5). The homogenate was filtered and centrifuged at 20,000 × *g* for 20 min. The resulting supernatant was re-centrifuged (Kontron Centrikon T-1180) at 190,000 × *g* for 60 min. The obtained precipitate was dissolved in 5 mL of Tris-HCl buffer (0.2 M, pH 7.4) and used as a microsomal enzyme solution. Cold acetone was added to the 190,000 × *g* supernatant and held at -25 °C for 20 min. The resulting precipitate was dissolved in 20 mL Tris-HCl buffer (0.2 M, pH 7.4) and used as a cytosolic enzyme.

The enzyme assay was carried out with an addition of [$^2H_6$]-CN to the microsomal enzyme solution (500 μL) at 37 °C for 30 min. After finishing the reaction, [$^2H_0$]-6α-hydroxyCN, -6-oxoCN and 6-deoxoCT were added to the reaction solution as internal standard for quantitative analyses. The reaction solution was charged onto ODS catridge (Sep-Pak C_{18}), and eluted with 0, 50 and 100% methanol. The 100% methanol fraction was further purified by a reversed phase HPLC (Shenshu Pak, Pegasil-D ODS, 10 × 150 mm) with 50% acetonitrile for 25 min and gradient to 100% acetonitrile for 35 min as a elution solvent. Finally, the enzyme products were analyzed by a capillary GC-SIM.

GC-MS/SIM analysis. GC-MS and GC-SIM analyses were conducted by Hewlett-Packard 5973 mass spectrometer (EI, 70 eV) connected to 6890 gas chromatograph fitted with a fused silica capillary column (HP-5, 0.25 × 30 m, 0.25 μm film thickness). GC condition was as follow; 1.5 mL He min^{-1}, on-column injection mode, oven temperature: 150 °C for 2 min, thermal gradient 40 min^{-1} to 280 °C, and then 280 °C. Before injection, enzyme products were treated with *N*-methyl-*N*-trimethylsilyltrifluroroacetamide at 80 °C for 30 min.

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