Metabolism of Typhasterol, a Brassinosteroid, in Suspension Cultured Cells of *Marchantia polymorpha*

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Brassinosteroids (BRs) are steroidal plant hormones which exert essential roles in normal growth and development of plants. Recently, biosynthesis of BRs in higher plants was intensively investigated by feeding experiments using isotope-labelled substrates and molecular genetic analyses of BR-deficient mutants.[1-5] Two parallel biosynthetic pathways, namely the early and late C6-oxidation pathway, to synthesis brassinolide (BL), the most active BR in plant kingdom, were established.[6-8] Although the occurrence of BRs in lower plants has also been demonstrated,[4,9] however, only limited information on biosynthesis of BRs in lower plants is available now. We have reported that cultured cells of *Marchantia polymorpha*, a liverwort, show a BR activity, and 24-methylcholesterol as a biosynthetic precursor for BL is contained in the cells. In addition, the presence of a partial sequence of the early C6-oxidation pathway, teasterone (TE) → 3-dehydroteasterone (3-DHT) → typhasterol (TY) was demonstrated in the *Marchantia* cells by *in vivo* feeding experiments and *in vitro* enzymatic conversions, suggesting that the early C6-oxidation may be included to produce BRs in the cells.[10] Our continuous interest in biosynthesis of BRs in the *Marchantia* cells led us to feed TY to the cells, and the fate of TY was investigated in this study.

Because endogenous level of BRs in suspension cultured cells of *M. polymorpha* (10 g) was negligible, TY was fed to the cells. After a 7-day incubation, the cells were collected and extracted with 80% methanol. The extracts were concentrated to aqueous residue *in vacuo*, combined with the culture medium, and purified by the methods in "Experimental Section". After a reversed phase HPLC, biological activities were found in the fractions referred as I, II, III and IV based on their polarities (Figure 1). Identification of the active compound in I, II, III and IV was carried out by full-scan GC-MS.

The retention time of III in HPLC corresponded to that of TY fed to the cells. Methaneboronate (MB)-trimethylsilyl (TMSi) ether of the active compound gave prominent ions at m/z 544 [M^+], 529, 515, 454, and 155 whose retention time on GC was identical to that of TY MB-TMSi ether (Table 1). Therefore, the active compound in III was identified to be TY. The most polar metabolite in I gave a molecular ion at m/z 512 after methaneboronation, indicating bismethaneboronate (BMB) of the metabolite has been formed. This suggested that a hydroxyl has been introduced at C2 of TY to produce castasterone (CS) BMB. GC retention time and other characteristic ions at m/z 441, 399, 358, 287 and 155 for BMB of the metabolite were the same as those of authentic CS BMB, demonstrating the metabolite in I was CS. As a MB-TMSi ether, II showed the same prominent ions at m/z 544[M^+], 529, 515, 454 and 155 as those of TY MB-TMSi ether, but intensities of the ions were different from the same ions shown in TY MB-TMSi ether (Table 1), suggesting that the metabolite in II is a stereoisomer of TY, most likely TE. The GC retention time of MB-TMSi ether of the metabolite in II was equal to that of authentic TE MB-TMSi ether, confirming the metabolite to be TE. The identification of TE as a metabolite of TY suggested that a conversion of TY to TE occurred in *Marchantia* cells. Thus, the less polar metabolite in IV appeared to be 3-DHT, a possible intermediate of the conversion. In fact, MB of the metabolite in IV showed identical GC retention time and prominent ions at m/z 470 [M^+], 399, 316, 298, 287, 245 and 155 to authentic 3-DHT MB, determining the metabolite in IV to be 3-DHT. In consequence, TE, 3-DHT and CS were identified as metabolite of TY from cultured cells of *M. polymorpha*.

2α-Hydroxylation from TY to CS is a reaction in which an oxygen atom is incorporated to the substrate. In many biological systems, the reaction is generally catalyzed by a cytochrome P450 (Cyt P450) monooxygenase which is associated to membrane of cells, most likely endoplasmic reticulum (E.R.) and needs O_2 and NADPH as a substrate and a cofactor, respectively.[11-15] To determine intracellular localization of the enzyme mediating the 2α-hydroxylation, namely TY 2α-hydroxylase, a microsomal and cytosolic enzyme solution were prepared from the *Marchantia* cells by ultracentrifugation. Activity of NADPH-cyt c reductase, a E.R. marker enzyme, in the microsomal solution was ca 28 times higher than that in the cytosolic enzyme solution, indicating microsomal enzymes in the cells were properly separated from cytosolic enzymes (Table 2).

The reaction by TY 2α-hydroxylase was carried out by addition of TY as a substrate to the standard assay mixture using the microsomal or cytosolic enzyme solution. After finishing the reaction, deuterium labelled product, [C26, 28-$^2H_6$]-CS, was added to the assay mixture as an internal standard for quantitative analysis. The assay mixture was then purified by the methods described in Experimental Section, and analyzed by GC-selected ion monitoring (SIM) after methaneboronation. BMB of the product showed characteristic ions at m/z 512 (M^+) and 155 (base peak) at the same

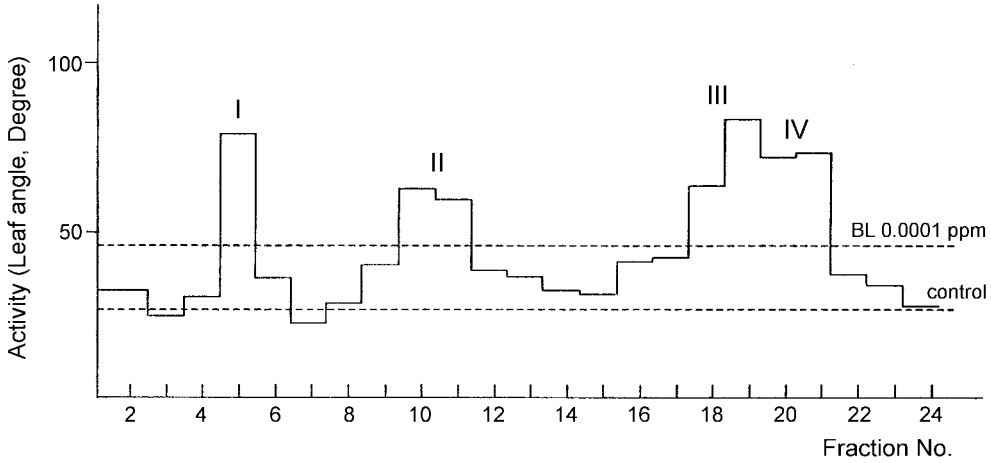

Figure 1. Distribution of biological activity determined by the rice lamina inclination assay after a reversed phase HPLC.

Table 1. GC-MS data for active compounds in a reversed phase HPLC (Fig. 1) and authentic BRs

Compound	Retention Time on GC	Prominent ions (m/z, relative intensity %)
I[a]	20.35	512(M^+, 76), 441(11), 399(19), 358(49), 287(35), 155(100)
II[b]	21.14	544(M^+, 22), 529(62), 515(100), 454(2), 155(6)
III[b]	17.33	544(M^+, 65), 529(43), 515(100), 454(65), 155(22)
IV[a]	20.48	470(M^+, 62), 399(8), 316(19), 298(9), 287(11), 245(16), 155(100)
Castasterone[a]	20.35	512(M^+, 76), 441(11), 399(19), 358(49), 287(35), 155(100)
Teasterone[b]	21.14	544(M^+, 23), 529(64), 515(100), 454(4), 155(8)
Typhasterol[b]	17.33	544(M^+, 63), 529(44), 515(100), 454(68), 155(24)
3-Dehydroteasterone[a]	20.48	470(M^+, 61), 399(7), 316(15), 298(8), 287(9), 245(15), 155(100)

[a]The sample was analyzed as a derivative of methaneboronate. [b]The sample was analyzed as a derivative of methaneboronate-trimethylsilyl ether.

Table 2. Activity for TY 2α-hydroxylase and NADPH-cyt c reductase in subcellular fractions prepared from cultured cells of *M. polymorpha*

Subcellular fraction	Enzyme activity	
	TY 2α-hydroxylase[a]	NADPH-cyt c reductase[b]
Cytosolic	0.06	0.10
Microsomal	1.51	2.80

[a]The enzyme activity was expressed as ng of the product mg protein^{-1} min^{-1}. [b]The enzyme activity was expressed as a specific activity, μmol of the product mg protein^{-1} min^{-1}.

GC retention time as that of authentic CS BMB, indicating that a conversion of TY to CS was successfully catalyzed by TY 2α-hydroxylase in the *Marchantia* cells (Figure 2). The specific activity for 2α-hydroxylase in the microsomal and cytosolic solution was 1.51 and 0.06 ng of the product mg protein^{-1} min^{-1}, respectively, indicating that activity for the enzyme is concentrated in the microsomal solution. Therefore, TY 2α-hydroxylase in the *Marchantia* cells was thought to be associated to the E.R. membrane.

We previously demonstrated that TE was converted into 3-DHT and TY by a two-step C3-epimerization mediated two cytosolic enzymes, TE dehydrogenase and 3-DHT reductase in *Marchantia* cells.[10] In this study, we identified 3-DHT and TE as metabolites of TY from *Marchantia* cells. This provided that a reverse C3-epimerization from TY to TE *via* 3-DHT also occurred, and the enzymes catalyzing the C3-

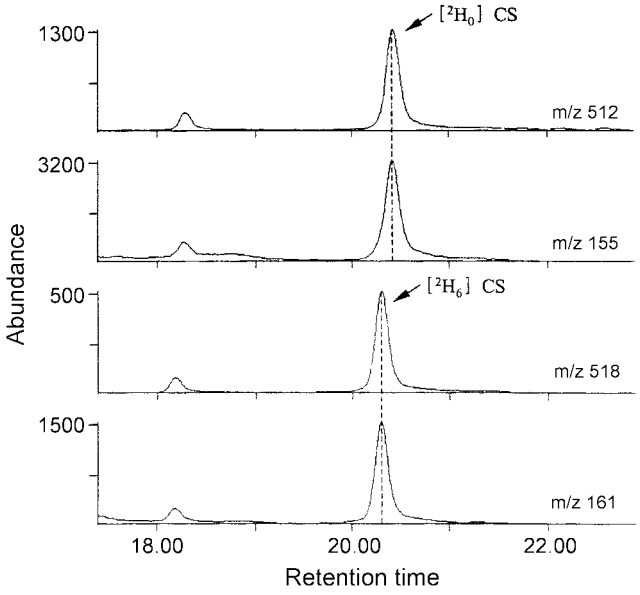

Figure 2. GC-SIM analysis of the product for TY 2α-hydroxylase in cultured cells of *M. polymorpha*.

epimerization, TE dehydrogenase and 3-DHT reductase are reversible enzymes in the cells (Figure 3). The reversible C3-epimerization was also found in higher plants. Therefore, the reversible activities of TE dehydrogenase and 3-DHT reductase are conserved during evolution from low to

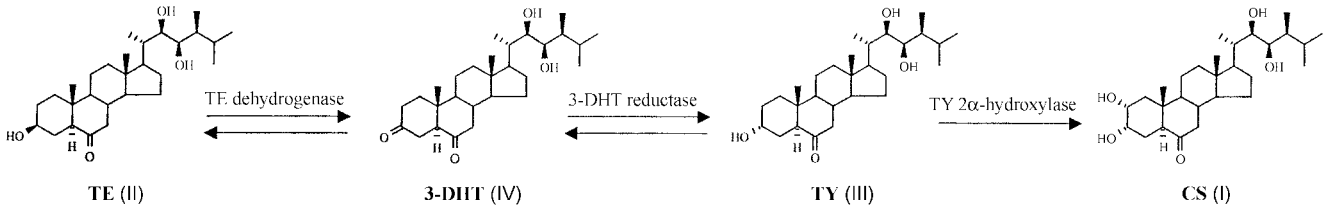

Figure 3. A partial sequence for BRs biosynthesis found in cultured cells of *M. polymorpha*.

higher plants.

In bioassays, TY showed much less biological activity than that of CS, indicating that C2α-hydroxylation of TY is important to express a strong BR activity. In *Phaseolus vulgaris*, TY 2α-hydroxylase was characterized to be a Cyt P450 by *in vitro* enzymatic analysis including a membrane association, requirements of O_2 and NADPH, inhibition by CO and Cyt P450 inhibitors, and a reversal activity of CO-induced inhibition by a blue light (Data will be published elsewhere). The *Marchantia* TY 2α-hydroxylase showed a membrane association and requirements of O_2 and NADPH for the activity, suggesting that it is also a Cyt P450 enzyme. To confirm that, characterization of TY 2α-hydroxylase in *Marchantia* cells as a Cyt P450 is now underway.

Experimental Section

Purification of metabolites. The 95% ethanolic solution (30 μL) of TY (100 μg) was added to the medium of the cell suspension culture at the 14th day of growth. After incubation for 7 days, the cells (10 g) were collected and extracted with 80% methanol (200 mL × 3). The extracts were concentrated to aqueous phase *in vacuo*, combined with the culture medium (150 mL), and subjected to a reversed phase chromatography (Merck, C_{18}). The biologically active 70, 80 and 90% methanol fractions were combined and chromatographed on Sephadex LH-20 column (bed volume 340 mL; 22 × 900 mm) using a 4 : 1 mixture of methanol-chloroform at a flow rate of 0.5 ml min^{-1}. The bioactive fractions with 0.65-0.75 of Ve/Vt were combined, dissolved in small volume of methanol, and subjected to a reversed phase HPLC (8 × 100 mm, 4 μm, Novapak C_{18} column) at a flow rate of 1 mL min^{-1} with 60% acetonitrile. Biologically active fraction I, II, III and IV were separately collected to be analyzed by full-scan GC-MS.

Bioassay. The rice lamina inclination assay using a cultivar Koshihikari was carried out to examine BRs activity.[16]

Enzyme preparation. Suspension cultured cells (50 g) of *M. polymorpha* were ground in a prechilled mortar and pestle in 80 mL of 0.1 M sodium phosphate (pH 7.4) containing 250 mM sucrose, 15 mM 2-mercaptoethanol, 1 mM EDTA, 40 mM ascorbate, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 15% (v/v) glycerol and 1% (w/v) insoluble polyvinylpyrrolidone. The homogenate was filtered and centrifuged at 15,000 × g for 30 min. The resulting supernatant was re-centrifuged at 190,000 × g for 120 min (Kontron Centrikon T-1180). The obtained pellet was re-suspended in 4 mL of 0.1 M sodium phosphate (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% (v/v) glycerol and used as a microsomal enzyme solution. Cytosolic enzymes were precipitated from the supernatant by addition of cold acetone to 40% (v/v) final concentration. The supernatant-acetone mixture was kept at -25 °C for 20 min and centrifuged at 13,000 × g for 10 min. The resulting precipitate was dissolved in 8 mL of 0.1 M sodium phosphate (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% (v/v) glycerol and used as a cytosolic enzyme solution.

Enzyme assays. The enzyme assays were performed in duplicate and the standard assay mixture consisted of 3 mg protein mL^{-1} from either cytosolic or microsomal enzymes in the re-suspension buffer, 0.8 mM NADPH and 5 μg of TY in a total volume of 1.2 mL. The reaction was initiated by addition of NADPH. The reaction mixture was incubated at 37 °C for 30 min with shaking. The reaction was terminated by addition of 1.2 mL of ethyl acetate and [C26, 28-$^2H_6$]-CS (50 ng) was added to the reaction mixtures as an internal standard for quantitative analysis. The ethyl acetate soluble fraction was concentrated *in vacuo*, dissolved in 50% methanol (5 mL), and charged on an ODS cartridge column (Sep-Pak Plus C_{18}, Waters). The fraction eluted with 100% methanol (5 mL) was concentrated *in vacuo* and subjected to a reversed phase HPLC (Novapak C_{18}, 8 × 100 mm) at a flow rate 1 mL min^{-1} with 45% acetonitrile for elution of the product for TY 2α-hydroxylase. The fractions corresponding to retention time of authentic CS (12.6 min) were collected and analyzed by GC-SIM after methaneboronation. To detect and calculate the amount of the product for TY 2α-hydroxylase, ions at m/z 512/518 (M^+) and 155/161 (base peak) characteristic for BMB of [$^2H_0$]/[$^2H_6$]-CS were monitored.

GC-MS/SIM analysis. GC-MS/SIM analyses were carried out with a Hewlett-Packard 5973 mass spectrometer (Electron impact ionization, 70 electron voltage) connected with 6890 gas chromatography fitted with a fused silica capillary column (HP-5, 0.25 mm × 30 m, 0.25 μm film thickness). GC oven temperature was maintained at 175 °C for 2 min, elevated to 280 °C at a rate of 40 °C min^{-1} and then maintained at 280 °C. The carrier gas was He at a flow rate of 1 mL min^{-1}, and the samples were introduced by an on-column injector. Prior to injection, the samples were treated with methaneboronic acid in pyridine (2 mg mL^{-1}) at 70 °C for 30 min to produce a bismethaneboronate.

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