Metabolism of Brassinolide in Suspension Cultured Cells of *Phaseolus vulgaris*

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Brassinosteroids (BRs) are steroidal plant hormones which are involved in growth and development of plants. In many biological systems, brassinolide (Figure 1) exhibits the highest biological activity among naturally-occurring BRs.[1-3] indicating that brassinolide is biologically active. Recently, two biosynthetic pathways to brassinolide, namely the early and late C-6 oxidation pathway, have been established in cultured cells of *Catharanthus roseus*.[4,5] These pathways seem to be ubiquitous in the plant kingdom because BRs belonging to these pathways have been observed in a variety of higher plants.[1,6-8] Recently, various BR-deficient mutants were isolated and found to have defects in the BR or sterol biosynthesis.[9,10]

However, very little is known about the metabolism of brassinolide. We earlier demonstrated that brassinolide fed to mung bean explants was deactivated to its 23-*O*-β-glucoside.[11] In explants and seedlings of rice, exogenously applied brassinolide was also metabolized to unknown polar metabolites that were water-soluble but non-glycosidic.[12] Recently, it was demonstrated that 24-epimers of brassinolide and castasterone are converted to various metabolites and their conjugates in suspension cultured cells of tomato[13,14] and *Ornithopus sativus*.[15] These findings prompted us to investigate the metabolism of brassinolide in cultured cells of *Phaseolus vulgaris* by feeding brassinolide as a substrate. The presence of brassinolide in *P. vulgaris* has been already demonstrated in the immature seed.[6]

The endogenous level of brassinolide in suspension cultured cells (100 g) of *P. vulgaris* was negligible. Thus, 95% ethanolic solution of brassinolide was supplied to the medium at 14th day of culture. After incubation for 7 days, the cultured cells were harvested and extracted with 80% methanol. The aqueous residue obtained from the extracts was combined with the culture medium before being subjected to a column of ODS. The fractions eluted with 70, 80 and 90% methanol exhibited biological activity in the rice lamina inclination assay. These fractions were combined, and then purified by a Sephadex LH-20 column chromatography. The biologically active fractions with 0.65-0.75 of the elution volume/total volume (Ve/Vt) were combined, and purified further by reversed phase HPLC. No significant biological activity except biological activity due to unmetabolized brassinolide fractions 32-34 was detected. However, silica gel thin layer chromatography suggested that fraction 19 contained a BR because of the presence of a blue-purple fluorescent spot characteristic for BRs at R_f 0.27. The compound in fraction 19 was derivatized into a bismethaneboronate (BMB) and analyzed by a capillary GC-MS.

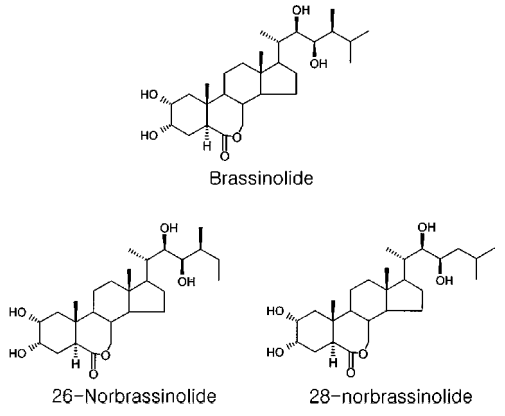

Figure 1. Structures of brassinosteroids.

BMB of the metabolite showed fragment ions at *m/z* 457, 374, 344, 177 and 163 which are characteristic of a 7-oxa-lactonic ring structure, indicating that the ring structure of the compound is identical with that of brassinolide (Figure 2). Further observed are a molecular ion at *m/z* 514 along with an ion at *m/z* 141 due to fission of C20-C22 and an ion at *m/z* 318 due to fission of B ring. These three ions were 14 mass reduced as compared with brassinolide BMB derivative, suggesting that either 26-methyl or 28-methyl was missing in the metabolite of brassinolide. Thus, the metabolite is suggested to be either 26-norbrassinolide or 28-norbrassinolide (Figure 1). 28-Norbrassinolide is a naturally-occurring BR which has already been synthesized. Direct GC-MS comparison of BMB of the metabolite and 28-norbrassinolide (Table 1) revealed that the metabolite is not 28-norbrassinolide but probably 26-norbrassinolide. 26-Norbrassinolide has been chemically synthesized (Suguru Takatsuto, unpublished data). Thus, 26-norbrassinolide was analyzed by GC-MS after methaneboronation. The obtained mass spectrum and GC retention time of 26-norbrassinolide BMB were exactly the same as those derived from BMB of the metabolite (Table 1). Therefore, the metabolite was determined to be 26-norbrassinolide.

Next, *in vitro* enzymatic conversion from brassinolide to

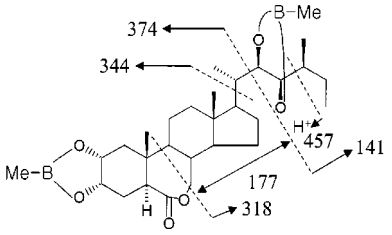

Figure 2. Mass fragmentation of the BMB of the metabolite o brassinolide fed to *P. vulgaris* cells.

Table 1. GC-MS Data of 28-Norbrassinolide, 26-Norbrassinolide and a Metabolite of Brassinolide Fed to *Phaseolus vulgaris* Cells

Compound[a]	RR_t in GC[b]	Prominent ions *m/z* (relative intensity, %)
28-Norbrassinolide	2.70	514 (M^+, 10), 457 (2), 374 (18), 344 (29), 318 (85), 177 (97), 163 (27), 141 (100)
26-Norbrassinolide	2.80	514 (M^+, 7), 457 (3), 374 (19), 344 (19), 318 (47), 177 (57), 163 (23), 141 (100)
Metabolite of brassinolide	2.80	514 (M^+, 6), 457 (3), 374 (20), 344 (19), 318 (50), 177 (61), 163 (22), 141 (100)

[a]Compound was analyzed as a derivative of bismethaneboronate. [b]Relative retention time (RR_t) with respect to 5α-cholestane (6.470 min).

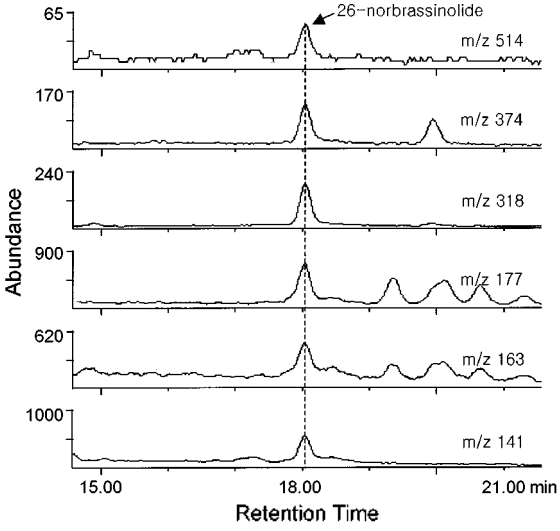

Figure 3. GC-selected ion monitoring analysis of the product mediated by brassinolide demethylase in *P. vulgaris* cells. Sample was analyzed as a bismethaneboronate. The relative retention time on GC of BMB of the enzyme product, 26-norbrassinolide, with respect to 5α-cholestane (6.450 min) was 2.80.

26-norbrassinolide by brassinolide demethylase, a tentative name of an enzyme responsible for the C26-demethylation of brassinolide, in *P. vulgaris* cells was examined. Cultured cells (20 g) were homogenized with 0.1 M sodium phosphate buffer (pH 7.4) and centrifuged at 15,000 × g for 20 min. The supernatant was re-centrifuged at 100,000 × g for 60 min. The resulting supernatant was precipitated with cold acetone, and used a crude enzyme solution. The enzyme assay was carried out in a reaction buffer (1 mL, 0.1 M sodium phosphate, pH 7.4) at 37 °C for 30 min by addition of brassinolide (5 μg). Then, the product was purified by reversed phase HPLC as described above, and analyzed by GC-selected ion monitoring (SIM) as a BMB derivative. As shown in Figure 3, selected ions at *m/z* 514, 374, 318, 177, 163 and 141 to identify 26-norbrassinolide were detected at the same GC retention time. Furthermore, the GC retention time was identical with that of authentic 26-norbrassinolide BMB. Therefore, conversion of brassinolide to 26-norbrassinolide by brassinolide demethylase in *Phaseolus* cells was demonstrated.

In yeast, rat and plants, steroidal demethylation at C-14 is known to proceed *via* C-14 hydroxymethyl and C-14 aldehyde.[16,17] Recently, hydroxylation at C-26 of brassinosteroids has been demonstrated to occur in tomato cell cultures, which convert 24-epibrassinolide and 24-epicastasterone into 26-hydroxy-24-epibrassinolide and 26-hydroxy-24-epicastasterone, respectively.[14,15] Thus, 26-norbrassinolide in *P. vulgaris* cells is expected to be formed *via* intermediates having either a hydroxyl, aldehyde or carboxyl group at C-26 (Figure 4). 26-Hydroxybrassinolide is essentially inactive in the rice lamina inclination bioassay.[18] We found that 26-norbrassinolide has less biological activity than that of brassinolide. Thus, hydroxylation and successive reactions leading to demethylation may be designated as a deactivation process of BRs in plants. Currently we are attempting to characterize the precursor(s) of 26-norbrassinolide unambiguously.

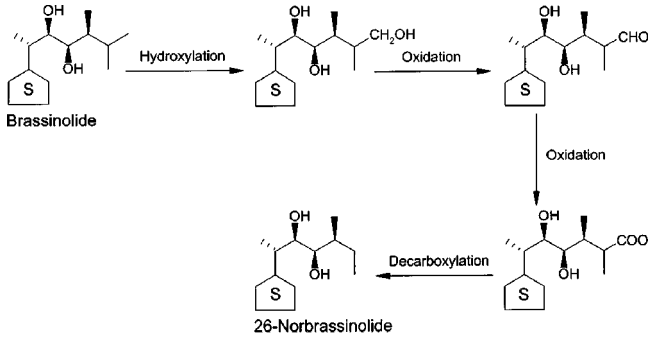

Figure 4. A possible scheme for brassinolide metabolism to 26-norbrassinolide in *P. vulgaris* cells. 'S' in the structure indicates the same ring structure as that of brassinolide.

Experimental Section

Isolation of a metabolite of brassinolide. The 95% ethanolic solution (30 μL) of brassinolide (15 μg) was added to the medium of the cell suspension culture of *Phaseolus vulgaris* at the 14th day of growth. After incubation for 7 days, the cells (100 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 ODS chromatography (bed volume 100 mL, Merck Lichroprep RP-18). The elution was carried out with the aqueous methanol increasing methanol content every 200 mL (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%). 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 33% acetonitrile. In HPTLC (Merck) developed with a 5 : 1 mixture of chloroform-methanol as solvent, fraction 19 showed a spot at R_f 0.27 after treatment with 70% aqueous sulfuric

acid and subsequent heating. The fraction 19 was concentrated and analyzed by GC-MS.

Enzyme preparation and assay. The cultured cells (20 g) of *Phaseolus vulgaris* were ground in a mortar and pestle at 4 °C with 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 15 mM 2-mercaptoethanol, 250 mM sucrose, 40 mM ascorbic acid and 15% glycerol (v/v). The homogenate was filtered and centrifuged (Jouan SR 20.22) at 15,000 × g for 20 min. The resulting supernatant was re-centrifuged (Kontron Centrikon T-1180) at 100,000 × g for 60 min. Cold acetone was added to the 100,000 × g supernatant (final acetone concentration was 40%), and kept at -25 °C for 10 min. After centrifugation at 13,000 × g for 5 min, the resulting precipitate was used as a crude enzyme solution.

The enzyme assay was carried out in a reaction buffer (1 mL, 0.1 M sodium phosphate containing 1.5 mM 2-mercaptoethanol, 0.8 mM NADPH and 30% glycerol, pH 7.4) at 37 °C for 30 min by addition of brassinolide (5 μg). After finishing the reaction, the assay mixture was charged onto an ODS cartridge (SepPak C_{18}), and then eluted with 0, 50 and 100% methanol. The fraction eluted with 100% methanol was purified further by a reversed phase HPLC using the same condition described. The fraction corresponding to retention time of 26-norbrassinolide was collected and subjected to GC-SIM.

GC-MS and GC-SIM analysis. The GC-MS and GC-SIM analyses were performed by Hewlett-Packard 5973 mass spectrometer (Electron Impact ionization, 70 electron voltage) 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: 1 mL He min^{-1}, on-column injection mode, oven temperature: 175 °C for 2 min, thermal gradient 40 °C min^{-1} to 280 °C, and then 280 °C. Prior to injection, the sample was treated with pyridine containing methaneboronic acid (0.5 mg mL^{-1}) at 80 °C for 30 min to produce a BMB derivative.

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