J Plant Growth Regul (2006) 25:195–202
DOI: 10.1007/s00344-006-0005-x

J P G R
Journal of Plant Growth Regulation

Metabolic Conversion of Castasterone and Brassinolide into Their Glucosides in Higher Plants

Kazuo Soeno,[1]* Shozo Fujioka,[1,2] Sayoko Hiranuma,[1] Hideharu Seto,[1,2] and Shigeo Yoshida[1,2]

[1]*RIKEN, Plant Science Center, Tsurumi, Yokohama 230-0045, Japan;* [2]*RIKEN, Discovery Research Institute, Wako-shi, Saitama 351-0198, Japan*

Abstract

Castasterone (CS) and brassinolide (BL) were administered to mung bean (*Vigna radiata*) explants, *Arabidopsis thaliana* seedlings, and cultured *Catharanthus roseus* cells, and the glucosylated metabolites were analyzed using LC/MS/MS. In mung bean and *C. roseus*, CS-2-*O*-glucoside (CS-2G), -3-*O*-glucoside (CS-3G), -22-*O*-glucoside (CS-22G), and -23-*O*-glucoside (CS-23G) were identified as metabolites of CS, whereas BL-2G, BL-3G, and BL-23G were identified as metabolites of BL. In *A. thaliana*, CS and BL were converted into their respective 2-*O*- and 23-*O*-glucosides. Of the metabolites identified with BL and CS administration, BL-23G was the predominant metabolite in mung bean and *A. thaliana*, whereas the 3-*O*-glucoside of BL was abundant in *C. roseus*. This is the first report of the metabolic conversion of CS into CS-2G, CS-3G, CS-22G, and CS-23G, and of BL into BL-2G and BL-3G. Our results indicate that the glucosylation profiles of BL and CS vary with plant species, and that the glucosylation of CS is rather limited quantitatively, compared with that of BL.

Key words: Brassinosteroid; Metabolism; Glucosylation; Mung bean; *Arabidopsis thaliana*; *Catharanthus roseus*; Brassinolide; Castasterone.

Introduction

Brassinosteroids (BRs) are important plant steroid hormones that regulate plant growth and development (Clouse 2002). Brassinosteroid biosynthesis has been well characterized and involves a complex set of pathways in which campesterol is converted into brassinolide (BL) via a series of reductions, hydroxylations, epimerizations, and oxidations (Fujioka and Yokota 2003). In contrast to our detailed understanding of BR biosynthesis, the mechanisms that control BR homeostasis remain largely unknown. The precise control of homeostasis is a prerequisite for the normal growth and development of higher plants. Various feeding experiments have shown that BRs applied to plants are conjugated with sugars, sulfuric acid, and fatty acids (Fujioka and Sakurai 1997; Yokota 1999; Fujioka and Yokota 2003). Glycosylation is one of the general metabolic processes that occur in plant hormone homeostasis. Although several BR glucosides have been isolated in metabolic studies using epimers of Castasterone (CS) and BL (Schneider and

Received: 24 January 2006; accepted: 21 March 2006; Online publication: 26 September 2006
*Corresponding author; e-mail: soeno@psc.riken.jp

others 1994; Hai and others 1995, 1996), little is known of the glucosylation of important BRs: BL and its biosynthetic precursor CS. Only BL-23-*O*-glucoside (BL-23G) has been identified as a metabolite of BL in mung bean explants (Suzuki and others 1993b). That study suggested that CS was converted into glucosides as minor components, although the existence of CS glucosides remains to be verified.

To better understand the glucosylation of BRs in higher plants, we investigated the metabolic conversion of BRs into glucosides. For this purpose, we synthesized the glucosides of CS and BL as reference standards. In this study, we report the glucosylation profiles of CS and BL in mung bean explants, *Arabidopsis thaliana* seedlings, and cultured *Catharanthus roseus* cells using LC/MS/MS analysis.

Materials and Methods

Chemicals

The synthesis and structure assignment of the standard BR-glucosides used in this study will be described elsewhere (Seto and others unpublished). Selected data of ^{1}H-nuclear magnetic resonance (NMR) spectra of these compounds are as follows. Chemical shifts were recorded as δ values in parts per million (ppm) relative to a peak of the solvent (CD_3OD) at 3.30 ppm as an internal reference. All *J*-values are given in Hz.

*CS-2G (Castasterone 2-*O*-β-*D*-glucopyranoside).* ^{1}H-NMR (500 MHz, CD_3OD) δ 0.72 and 0.76 (each 3H, each s, 18- and 19-H_3), 0.83 (3H, d, *J* = 6.9, 28-H_3), 0.90 (3H, d, *J* = 6.9, 21-, 26- or 27-H_3), 0.93 (3H, d, *J* = 6.4, 21-, 26- or 27-H_3), 0.96 (3H, d, *J* = 6.9, 21-, 26- or 27-H_3), 2.12 (1H, d, *J* = 12.3, 7-H), 2.20 (1H, dd, *J* = 12.3 and 4.6, 7-H), 2.75 (1H, dd, *J* = 12.4 and 3.2, 5-H), 3.18 (1H, dd, *J* = 9.2 and 7.8, Glc 2-H), 3.30 (2 × 1H, Glc 4- and Glc 5-H), 3.35 (1H, t, *J* = 9.2, Glc 3-H), 3.51 (1H, d, *J* = 8.3, 22-H), 3.66 (1H, dd, *J* = 11.9 and 5.0, Glc 6-H), 3.67 (1H, d, *J* = 8.3, 23-H), 3.83 (1H, m, 2-H), 3.83 (1H, dd, *J* = 11.9 and 1.8, Glc 6-H), 4.15 (1H, br s, 3-H), 4.37 (1H, d, *J* = 7.8, Glc 1-H).

*CS-3G (Castasterone 3-*O*-β-*D*-glucopyranoside).* ^{1}H-NMR (500 MHz, CD_3OD) δ 0.72 and 0.76 (each 3H, each s, 18- and 19-H_3), 0.83 (3H, d, *J* = 6.9, 28-H_3), 0.90 (3H, d, *J* = 6.9, 21-, 26- or 27- H_3), 0.93 (3H, d, *J* = 6.4, 21-, 26- or 27-H_3), 0.96 (3H, d, *J* = 6.9, 21-, 26- or 27- H_3), 2.19 (1H, dd, *J* = 12.8 and 5.0, 7-H), 2.80 (1H, dd, *J* = 12.4 and 2.8, 5-H), 3.21 (1H, m, Glc 5-H), 3.22 (1H, dd, *J* = 8.7 and 7.8, Glc 2-H), 3.30 (1H, dd, *J* = 9.6 and 8.2, Glc 4-H), 3.35 (1H, t, *J* = 8.7, Glc 3-H), 3.51 (1H, dd, *J* = 8.3 and 1.4, 22-H), 3.60–3.70 (3 × 1H, 2-, 23- and Glc 6-H), 3.75 (1H, dd, *J* = 11.9 and 2.3, Glc 6-H), 4.00 (1H, br s, 3-H), 4.41 (1H, d, *J* = 7.8, Glc 1-H).

*CS-22G (Castasterone 22-*O*-β-*D*-glucopyranoside).* ^{1}H-NMR (500 MHz, CD_3OD) δ 0.72 and 0.75 (each 3H, each s, 18- and 19-H_3), 0.86 (3H, d, *J* = 6.4, 28-H_3), 0.93 (3H, d, *J* = 6.9, 21-, 26- or 27-H_3), 0.96 (3H, d, *J* = 6.9, 21-, 26- or 27-H_3), 0.99 (3H, d, *J* = 6.9, 21-, 26- or 27-H_3), 2.09 (1H, d, *J* = 12.8, 7-H), 2.20 (1H, dd, *J* = 12.8 and 5.0, 7-H), 2.71 (1H, dd, *J* = 12.4 and 3.2, 5-H), 3.24 (1H, dd, *J* = 8.7 and 7.8, Glc 2-H), 3.29–3.46 (3 × 1H, Glc 3-, Glc 4- and Glc 5-H), 3.56 (1H, d, *J* = 7.8, 22-H), 3.62–3.72 (2 × 1H, 2- and Glc 6-H), 3.81 (1H, dd, *J* = 7.8 and 1.8, 23-H), 3.86 (1H, dd, *J* = 12.4 and 1.8, Glc 6-H), 3.94 (1H, br s, 3-H), 4.33 (1H, d, *J* = 7.8, Glc 1-H).

*CS-23G (Castasterone 23-*O*-β-*D*-glucopyranoside).* ^{1}H-NMR (500 MHz, CD_3OD) δ 0.71 and 0.75 (each 3H, each s, 18- and 19-H_3), 0.91 (3H, d, *J* = 6.4, 21-, 26-, 27- or 28-H_3), 0.91 (3H, d, *J* = 6.9, 21-, 26-, 27- or 28-H_3), 0.92 (3H, d, *J* = 6.4, 21-, 26-, 27- or 28-H_3), 0.97 (3H, d, *J* = 6.4, 21-, 26-, 27- or 28-H_3), 2.09 (1H, m, 7-H), 2.20 (1H, dd, *J* = 13.3 and 5.0, 7-H), 2.72 (1H, dd, *J* = 11.9 and 3.2, 5-H), 3.21 (1H, t, *J* = 7.8, Glc 2-H), 3.30 (3 × 1H, Glc 3-, Glc 4- and Glc 5-H), 3.63–3.69 (3 × 1H, 2-, 22- and Glc 6-H), 3.80 (1H, d, *J* = 8.3, 23-H), 3.87 (1H, d, *J* = 11.0, Glc 6-H), 3.94 (1H, br s, 3-H), 4.32 (1H, d, *J* = 7.8, Glc 1-H).

*BL-2G (Brassinolide 2-*O*-β-*D*-glucopyranoside).* ^{1}H-NMR (600 MHz, CD_3OD) δ 0.75 (3H, s, 18-H_3), 0.83 (3H, d, *J* = 7.1, 28-H_3), 0.89 (3H, d, *J* = 6.6, 21-H_3), 0.90 (3H, s, 19-H_3), 0.93 (3H, d, *J* = 7.1, 26- or 27-H_3), 0.96 (3H, d, *J* = 6.6, 26- or 27-H_3), 3.18 (1H, dd, *J* = 9.1 and 7.6, Glc 2-H), 3.22 (1H, dd, *J* = 12.1 and 4.5, 5-H), 3.28 (1H, m, Glc 5-H), 3.30 (1H, m, Glc 4-H), 3.35 (1H, dd, *J* = 9.1 and 8.6, Glc 3-H), 3.50 (1H, dd, *J* = 9.1 and 1.5, 22-H), 3.67 (1H, dd, *J* = 12.0 and 4.6, Glc 6-H), 3.67 (1H, dd, *J* = 9.1 and 2.0, 23-H), 3.76 (1H, ddd, *J* = 12.1, 4.0 and 2.5, 2-H), 3.84 (1H, dd, *J* = 12.0 and 2.0, Glc 6-H), 4.14 (1H, ddd, *J* = 4.0, 2.5 and 2.5, 3-H), 4.08 (1H, dd, *J* = 12.6 and 1.0, 7-H), 4.19 (1H, dd, *J* = 12.6 and 9.1, 7-H), 4.38 (1H, d, *J* = 7.6, Glc 1-H).

*BL-3G (Brassinolide 3-*O*-β-*D*-glucopyranoside).* ^{1}H-NMR (600 MHz, CD_3OD) δ 0.75 (3H, s, 18-H_3), 0.83 (3H, d, *J* = 7.1, 28-H_3), 0.89 (3H, d, *J* = 6.6, 21-H_3), 0.90 (3H, s, 19-H_3), 0.93 (3H, d, *J* = 7.1, 26- or 27-H_3), 0.96 (3H, d, *J* = 6.6, 26- or 27-H_3), 3.25 (1H,

dd, J = 9.1 and 7.6, Glc 2-H), 3.25 (1H, ddd, J = 9.1, 5.1 and 2.5, Glc 5-H), 3.31 (1H, dd, J = 9.1 and 8.6, Glc 4-H), 3.33 (1H, dd, J = 12.1 and 4.5, 5-H), 3.36 (1H, dd, J = 9.1 and 8.6, Glc 3-H), 3.50 (1H, dd, J = 8.1 and 1.0, 22-H), 3.63 (1H, ddd, J = 12.6, 4.5 and 2.5, 2-H), 3.66 (1H, dd, J = 11.6 and 5.1, Glc 6-H), 3.67 (1H, dd, J = 8.1 and 1.5, 23-H), 3.78 (1H, dd, J = 11.6 and 2.5, Glc 6-H), 3.97 (1H, ddd, J = 4.0, 2.5 and 2.0, 3-H), 4.06 (1H, dd, J = 12.6 and 1.0, 7-H), 4.24 (1H, dd, J = 12.6 and 9.6, 7-H), 4.42 (1H, d, J = 7.6, Glc 1-H).

*BL-22G (Brassinolide 22-*O*-β-*D*-glucopyranoside).* ^{1}H-NMR (600 MHz, CD_3OD) δ 0.75 (3H, s, 18-H_3), 0.86 (3H, d, J = 6.6, 28-H_3), 0.89 (3H, s, 19-H_3), 0.93 and 0.96 (each 3H, each d, J = 6.6, 26- and 27-H_3), 0.98 (3H, d, J = 7.1, 21-H_3), 3.20 (1H, dd, J = 12.6 and 4.5, 5-H), 3.23 (1H, dd, J = 9.1 and 7.6, Glc 2-H), 3.32 (1H, m, Glc 5-H), 3.33 (1H, m, Glc 4-H), 3.36 (1H, m, Glc 3-H), 3.55 (1H, br d, J = 8.6, 22-H), 3.59 (1H, ddd, J = 12.6, 4.5 and 2.5, 2-H), 3.68 (1H, dd, J = 12.6 and 5.9, Glc 6-H), 3.81 (1H, dd, J = 8.6 and 2.0, 23-H), 3.86 (1H, dd, J = 12.6 and 2.0, Glc 6-H), 3.91 (1H, ddd, J = 4.0, 2.5 and 2.0, 3-H), 4.08 (1H, dd, J = 12.1 and 1.0, 7-H), 4.17 (1H, dd, J = 12.1 and 9.1, 7-H), 4.32 (1H, d, J = 7.6, Glc 1-H).

*BL-23G (Brassinolide 23-*O*-β-*D*-glucopyranoside).* ^{1}H-NMR (600 MHz, CD_3OD) δ 0.75 (3H, s, 18-H_3), 0.89 (3H, s, 19-H_3), 0.90 (3H, d, J = 6.6, 21-H_3), 0.92 and 0.97 (each 3H, each d, J = 6.6, 26- and 27-H_3), 0.92 (3H, d, J = 6.6, 28-H_3), 3.21 (1H, dd, J = 9.1 and 7.7, Glc 2-H), 3.22 (1H, dd, J = 12.1 and 4.4, 5-H), 3.33 (1H, m, Glc 4-H), 3.33 (1H, m, Glc 5-H), 3.34 (1H, m, Glc 3-H), 3.63 (1H, m, 2-H), 3.63 (1H, br d, J = 7.7, 22-H), 3.67 (1H, dd, J = 12.1 and 1.1, Glc 6-H), 3.80 (1H, br d, J = 7.7, 23-H), 3.87 (1H, dd, J = 12.1 and 4.4, Glc 6-H), 3.95 (1H, m, 3-H), 4.08 (1H, br d, J = 12.1, 7-H), 4.20 (1H, dd, J = 12.1 and 9.4, 7-H), 4.32 (1H, d, J = 7.7, Glc 1-H).

Analysis

LC-TOF/MS and LC/MS/MS analyses were carried out on a QSTAR Pulsar hybrid quadrupole TOF mass spectrometer (PE Sciex/Applied Biosystems) equipped with a Turbo Ion Spray source connected to an HP 1100 Series HPLC System (Hewlett Packard/Agilent Technologies). Turbo Ion Spray source, a variant of the electospray ionization (ESI) MS interface, consists of the Ion Spray probe used in conjunction with a heated Turbo Probe, which blows a jet of heated dry N_2 gas (up to approximately 500°C) at the spray produced by the Ion Spray. TOF/MS and product ion scan (MS/MS) were performed in positive ion mode. The mass spectrometer settings were as follows: nebulizer gas, 50; turbo gas, 80; curtain gas, 45; ion spray voltage, 5.5 kV; ion source temperature, 450°C; and collision cell energies, 17.5 V for CS-Gs and 15 V for BL-Gs. The amounts of metabolic BR-Gs formed in plants were calculated from the ratio of the peak areas at m/z 465.3 (CS-Gs) and 481.3 (BL-Gs) from the product ion scan using the calibration curves of standard compounds.

Metabolism of Castasterone and Brassinolide in Mung Bean Explants: Experiment 1. Mung bean seeds were germinated in water overnight and grown in vermiculite for 3 days at 28°C under 16 h of daily illumination with fluorescent light. Seedlings with 1.2 ± 0.5 cm epicotyls were selected and decapitated at the hypocotyl 4 cm below the cotyledon, and the cotyledons were removed. A total of 175 explants were placed in 300-ml beakers containing 10^{-5} M CS or BL in half-strength MS medium supplemented with 1% sucrose. They were incubated for 5 days under the same conditions. Half-strength MS medium containing 10^{-5} M CS or BL was added daily, so that the explants absorbed a total of approximately 300 ml each. The explants were extracted with MeOH (100 ml twice) and partitioned between EtOAc (100 ml × 3) and water (100 ml). The EtOAc-soluble fraction was purified using a silica gel cartridge (Sep-Pak Vac Silica, 10 g; Waters) and eluted with $CHCl_3$ (100 ml), $CHCl_3$-MeOH (98:2, 100 ml), $CHCl_3$-MeOH (93:7, 100 ml), and $CHCl_3$-MeOH (7:3, 100 ml). The eluent with 30% MeOH was chromatographed on an aluminium oxide column (aluminium oxide 90 standardized, 10 g, Merck) eluted with MeOH (20 ml) and MeOH-H_2O (1:1, 20 ml). The eluent with 50% MeOH was subjected to an ODS cartridge (Sep-Pak Plus C_{18}, 300 mg; Waters) with MeOH-H_2O (1:1, 5 ml), MeOH-H_2O (8:2, 5 ml), and MeOH (5 ml). The eluent with 80% MeOH was subjected to ODS-HPLC (Cosmosil 5C_{18}-MS-II, 4.6 × 150 mm, Nacalai Tesque) at a flow rate of 0.52 ml/min with 45% MeCN. The fraction was collected at Rt 3.5–12.5 min, and then subjected to LC/MS/MS analysis. The settings for the LC section were as follows: Cosmosil 5C_{18}-MS-II (2 × 150 mm, Nacalai Tesque), 40% MeCN for CS-Gs and 35% MeCN for BL-Gs as the mobile phase at a flow rate of 0.2 ml/min, column compartment temp. 35°C.

Metabolism of Castasterone and Brassinolide in Mung Bean Explants: Experiment 2. In the second experiment, 200 mung bean explants with 2.0 ± 0.5 cm epicotyls and 4 cm hypocotyls were prepared from seedlings grown for 6 days at 21°C under a 16-h

day. One explant was placed in each 1.5-ml vial with/without 10^{-6} M CS or BL in 1 ml of half-strength MS medium supplemented with 1% sucrose for 3 days under the same conditions. The CS- or BL-treated explants (100 cuttings each) were divided into hypocotyls, epicotyls, and leaves with petioles. The fresh weights were as follows (CS/BL treatment): hypocotyls (9.6/10.8 g), epicotyls (3.7/7.2 g), and leaves (8.8/8.3 g). The tissues were extracted with MeOH, and partitioned between EtOAc (50 ml × 3) and water (50 ml). The EtOAc-soluble fraction was purified using a silica gel cartridge (Sep-Pak Vac Silica, 2 g; Waters) and eluted with $CHCl_3$-MeOH (93:7, 30 ml) and $CHCl_3$-MeOH (7:3, 30 ml). The eluent with 30% MeOH was purified using an ODS cartridge (Sep-Pak Plus C_{18}, 300 mg; Waters) with MeOH (10 ml) and subjected to ODS-HPLC (Pegasil ODS, 20 × 250 mm, Senshu Scientific) using 90% MeCN as the mobile phase at a flow rate of 8 ml/min. The fraction was collected at Rt 6–9 min, and subjected to LC/MS/MS analysis. The settings for the LC section were as follows: Pegasil ODS (2 × 150 mm, Senshu Scientific), 38% MeCN as the mobile phase at a flow rate of 0.1 ml/min, column compartment temperature, 35°C.

Metabolism of Castasterone and Brassinolide in **Arabidopsis thaliana** *Seedlings.* *Arabidopsis thaliana* (wild-type: Columbia) seedlings were germinated and grown on half-strength MS medium containing 1% agar and 1% sucrose under continuous light at 21°C. After 7 days, 15 seedlings per flask were transferred to 200-ml flasks containing 30 ml of half-strength MS medium supplemented with 1% sucrose. The seedlings were incubated at 21°C in the light on a rotary shaker (100 rpm). After 7 days in culture, an EtOH solution of CS or BL (1 μg/μl) was added to each flask to give a final concentration of 10^{-6} M, and the seedlings were incubated for 1 to 3 days under the same conditions. To identify and quantify the BR-Gs, 180 seedlings (CS treatment; 24.6 g fw, BL treatment; 27.6 g fw) were pooled, extracted, purified, and analyzed according to the methods described in the second mung bean experiment. In the time-course study, each flask of 15 seedlings was harvested for extraction, and purified according to the following modified procedure. The extract was partitioned between EtOAc (10 ml once, 5 ml twice) and water (10 ml). The EtOAc-soluble fraction was loaded on a silica gel cartridge (Sep-Pak Vac RC Silica, 500 mg; Waters) with $CHCl_3$-MeOH (93:7, 10 ml) and $CHCl_3$-MeOH (7:3, 10 ml). The 30% MeOH fraction was purified using an ODS cartridge (Sep-Pak Plus C_{18}, 300 mg; Waters) and analyzed using LC/MS/MS. The settings for the LC section were the same as in the second mung bean metabolism experiment.

Metabolism of Castasterone and Brassinolide in Cultured **C. roseus** *Cells.* Cultured *C. roseus* cells (V208) were grown in MS medium supplemented with 3% sucrose at 27°C with shaking at 100 rpm in the dark. The EtOH solution of CS or BL was added aseptically to a 200-ml flask containing cultured cells at log phase (8 days old) in 60 ml of MS medium to give a final concentration of 10^{-6} M. After 1-, 2-, 3-, or 7-day incubations, cultures were extracted with MeOH. The extract of a tenth part was purified and analyzed according to the method described for the time course study of the BL-23G levels in the *A. thaliana* experiments. After a 7-day incubation, the fresh weight of the cultured cells was approximately 20 g.

Results and Discussion

Optimization of Product Ion Scan (MS/MS) Analyses for BR-Gs

As reference standards, we synthesized the following glucosides: CS-2-*O*-glucoside (CS-2G), CS-3-*O*-glucoside (CS-3G), CS-22-*O*-glucoside (CS-22G), CS-23-*O*-glucoside (CS-23G), BL-2-*O*-glucoside (BL-2G), BL-3-*O*-glucoside (BL-3G), BL-22-*O*-glucoside (BL-22G), and BL-23-*O*-glucoside (BL-23G). Using these standards, an efficient LC/MS/MS detection method was developed, permitting direct monitoring of the metabolites of BL and CS in our metabolic studies.

To facilitate the identification of glucosylated BR metabolites, the product ion scan (MS/MS) settings were optimized using reference standards. The nebulizer, turbo jet, curtain gas flows, ion spray voltage, and temperature were optimized for TOF/MS scan mode, resulting in the detection of $[M + 18]^+$ ions together with quasimolecular ions $[M + H]^+$ of CS-Gs and BL-Gs. The product ion scan analyses done for $[M + 18]^+$ showed high sensitivity compared with those for $[M + H]^+$ for both BL-Gs and CS-Gs standards. Therefore, product ion scan analyses were performed at scans of *m/z* 644.4 (for CS-Gs) and 660.4 (for BL-Gs). The collision cell energies of the product ion scan were also optimized for CS-Gs (17.5 V) and BL-Gs (15 V) using the same setup. The mass spectra of CS-Gs and BL-Gs showed simple fragment patterns: $[M + 18]^+$, $[M + H]^+$ (quasimolecular ion), $[\text{aglycone} + H]^+$ (base peak), and the expulsion of nH_2O (Table 1). The recovery of BR-Gs using

Table 1. LC/MS/MS Data for Authentic BR-Gs and Metabolic BR-Gs Formed in Mung Bean Explants

			Mass spectrum (% relative intensity)[a]				
BR-Gs	*Rt*[b]	Sample	$[M + 18]^+$	$[M + H]^+$	$[aglycone + H]^+$	$[aglycone + H\text{-}H_2O]^+$	$[aglycone + H\text{-}2(H_2O)]^+$
CS-2G	4.90	Standard	644.4 (47.4)	627.4 (5.7)	465.3 (100)	447.3 (9.2)	429.3 (1.8)
	4.90	Metabolite	644.4 (50.1)	627.4 (4.8)	465.3 (100)	447.3 (8.6)	429.3 (1.8)
CS-3G	8.30	Standard	644.4 (30.3)	627.4 (8.3)	465.3 (100)	447.3 (6.2)	429.3 (1.6)
	8.30	Metabolite	644.4 (40.6)	627.4 (15)	465.3 (100)	447.3 (8.8)	429.3 (2.5)
CS-22G	6.60	Standard	644.4 (11.3)	627.4 (60.6)	465.3 (100)	447.3 (11.7)	429.3 (7.6)
	6.58	Metabolite	644.4 (20.3)	627.4 (64.1)	465.3 (100)	447.3 (10.7)	429.3 (11.7)
CS-23G	4.00	Standard	644.4 (5.4)	627.4 (64.6)	465.3 (100)	447.3 (11.0)	429.3 (7.7)
	3.98	Metabolite	644.4 (4.4)	627.4 (67.6)	465.3 (100)	447.3 (13.2)	429.3 (8.1)
BL-2G	9.14	Standard	660.4 (14.0)	643.4 (43.4)	481.3 (100)	463.3 (2.1)	445.3 (2.8)
	9.24	Metabolite	660.4 (14.7)	643.4 (35.1)	481.3 (100)	463.3 (2.2)	445.3 (4.7)
BL-3G	15.82	Standard	660.4 (11.0)	643.4 (4.3)	481.3 (100)	463.3 (0.5)	445.3 (2.0)
	15.82	Metabolite	660.4 (11.1)	643.4 (4.9)	481.3 (100)	463.3 (1.1)	445.3 (1.6)
BL-22G	12.68	Standard	660.4 (5.1)	643.4 (30.8)	481.3 (100)	463.3 (0.2)	445.3 (8.0)
		Metabolite (nd)					
BL-23G	6.74	Standard	660.4 (3.6)	643.4 (39.2)	481.3 (100)	463.3 (4.2)	445.3 (9.0)
	6.81	Metabolite	660.4 (2.2)	643.4 (34.9)	481.3 (100)	463.3 (3.0)	445.3 (7.0)

[a] *The intensity of the base peak was set at 100%.*
[b] *The mobile phase of the LC section used was 40% MeCN for CS-Gs analyses and 35% MeCN for BL-Gs analyses at a flow rate of 0.2 ml* min^{-1}.
nd: not detected.

reference standards (10 ng each, $n = 3$) in methods of mung bean experiment 2 and time-course study of *A. thaliana* experiment was as follows: BL-Gs; 19.8 ± 1.3% (BL-23G)–28.7 ± 2.8% (BL-3G) and 21.5 ± 0.5% (BL-23G)–32.4 ± 0.2 % (BL-3G), CS-Gs; 24.3 ± 3.7% (CS-2G)–56.1 ± 0.5% (CS-3G) and CS-Gs; 28.5 ± 1.2% (CS-2G)–67.5 ± 1.7% (CS-3G), respectively. However, neither stable isotopes nor reference compounds were used as internal standards in this study, thus the amounts of metabolized BR-glucosides were estimated roughly from calibration curves established for authentic compounds. The calibration curves showed good linearity (CS-Gs; $R^2 = 0.9954$–0.9999, BL-Gs; $R^2 = 0.9980$–1.000) in a dynamic range of 0.1–2.0 ng (CS-Gs) and 0.25–2.0 ng (BL-Gs).

Metabolism of Castasterone and Brassinolide in Mung Bean. To determine the metabolic conversion of CS and BL to their glucosides, CS and BL were administered to mung bean explants. In the first experiment with/without 10^{-5} M CS or BL, the mung bean explants were harvested 3 days after application, and extracted with methanol. The extract was partitioned between ethyl acetate and water. The ethyl acetate-soluble fraction was purified using aluminum oxide column chromatography, a C_{18} cartridge, and ODS-HPLC. The HPLC-purified fractions were analyzed using LC/MS/MS. No glucoside was detected in the control extract. With CS feeding, four CS-glucosides (CS-2G, CS-3G, CS-22G, and CS-23G) were detected, whereas with BL feeding, three BL-glucosides (BL-2G, BL-3G, and BL-23G) were detected (Table 1). The identification was based on retention times and mass spectra, by the direct comparison of standards and metabolites. Therefore, these glucosides were definitely identified in the LC/MS/MS analysis. Although the metabolic conversion of BL into BL-23G has been demonstrated previously (Suzuki and others 1993b), this study is the first to demonstrate the conversion of CS into CS-2G, CS-3G, CS-22G, and CS-23G, and BL into BL-2G and BL-3G. In addition, with CS feeding, a peak for a CS-glucoside-like substance was observed at a retention time of 5.73 min. The mass spectral data were as follows: *m/z* (rel. int.) 644.4 $[M + 18]^+$, (34.8), 627.4 $[M + H]^+$ (13.4), 465.3 $[aglycone + H]^+$ (100), 447.3 $[465.3\text{-}H_2O]^+$ (4.5), 429.3 $[447.3\text{-}H_2O]^+$ (3.6). The retention time and mass spectrum suggest that this glucoside formed after epimerization of a hydroxy group, probably at C-2 or C-3 of CS.

The second experiment involved more detailed administration experiments. The metabolic BR-glucosides identified in this study are summarized in Tables 2 and 3. Although CS-23G was a major metabolic glucoside with CS feeding, the conversion ratio of CS into all CS glucosides was only 0.023%. Therefore, only a trace amount of CS was glucosylated in mung bean explants. By

Table 2. CS-G Levels in Mung Bean Explants, *Arabidopsis thaliana* Seedlings, and Cultured *Catharanthus roseus* Cells

	Mung bean[a]				
	Leaf (8.8 g fw)	Epicotyl (3.7 g fw)	Hypocotyl (9.6 g fw)	*A. thaliana* (24.6 g fw)[b]	*C. roseus* (ca. 20 g fw)[c]
	pmol/g fresh weight (conversion rate, %)				
CS-2G	nd	nd	0.31 (0.003)	0.06 (0.0003)*	0.55 (0.02)
CS-3G	nd	nd	0.16 (0.002)	nd	2.26 (0.08)
CS-22G	nd	nd	0.69 (0.007)	nd	0.54 (0.02)*
CS-23G	nd	nd	1.21 (0.011)	0.26 (0.002)	2.25 (0.08)

**The value was extrapolated because the measured area was outside the range of the calibration curves.*
[a]100 nmol of CS were applied to 100 segments in total.
[b]360 nmol of CS were applied to 180 seedlings in total.
[c]60 nmol of CS were added per flask.
nd: not detected.

Table 3. BL-G Levels in Mung Bean Explants, *A. thaliana* Seedlings, and Cultured *C. roseus* Cells

	Mung Bean[a]				
	Leaf (8.3 g fw)	Epicotyl (7.2 g fw)	Hypocotyl (10.8 g fw)	*A. thaliana* (27.6 g fw)[b]	*C. roseus* (ca. 20 g fw)[c]
	pmol/g fresh weight (conversion rate, %)				
BL-2G	Nd	nd	4.96 (0.05)*	4.76 (0.04)*	0.95 (0.03)*
BL-3G	Nd	nd	4.12 (0.04)*	nd	9.19 (0.31)
BL-22G	Nd	nd	nd	nd	nd
BL-23G	0.35 (0.003)	17.6 (0.13)	2675 (28.94)	93.3 (0.71)	1.14 (0.04)*

**The value was extrapolated because the measured area was outside the range of the calibration curves.*
[a]100 nmol of BL were applied to 100 segments in total.
[b]360 nmol of BL were applied to 180 seedlings in total.
[c]60 nmol of BL were added per flask.
nd: not detected.

contrast, approximately 30% of the BL applied to mung bean explants was converted into BL-23G. Interestingly, most of the BL-23G was detected in hypocotyls (28.9%), whereas there was only a trace in epicotyls (0.13%) and leaves (0.003%). CS-2G, CS-3G, CS-22G, CS-23G, BL-2G, and BL-3G were also detected from hypocotyls, but not from epicotyls or leaves. These results suggest that only very small percentages of glucosides are transported acropetally.

Metabolism of Castasterone and Brassinolide in **A. thaliana** *Seedlings.* To test whether CS and BL are converted into glucosides in other higher plants, besides mung bean, the metabolism of CS and BL was examined in *A. thaliana* seedlings. In the first experiment, seedlings grown in a liquid medium were harvested 3 days after the application of BR, and the metabolites were analyzed using LC/MS/

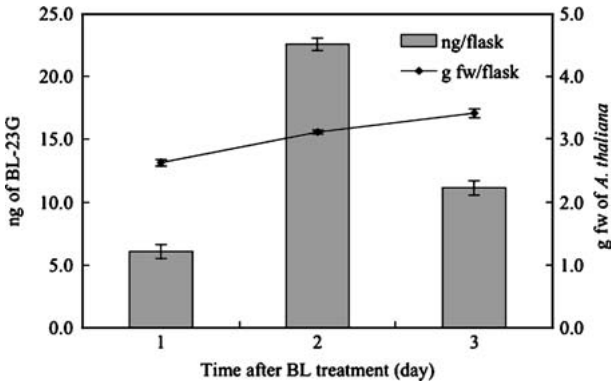

Figure 1. Change in the BL-23-*O*-glucoside levels and fresh weight of *Arabidopsis thaliana* seedlings after brassinolide (BL) treatment. The values are the mean BL-23-*O*-glucoside levels and fresh weights of *A. thaliana* seedlings measured in three independent experiments. Brassinolide (10^{-6} M) was added to a flask containing 15 seedlings.

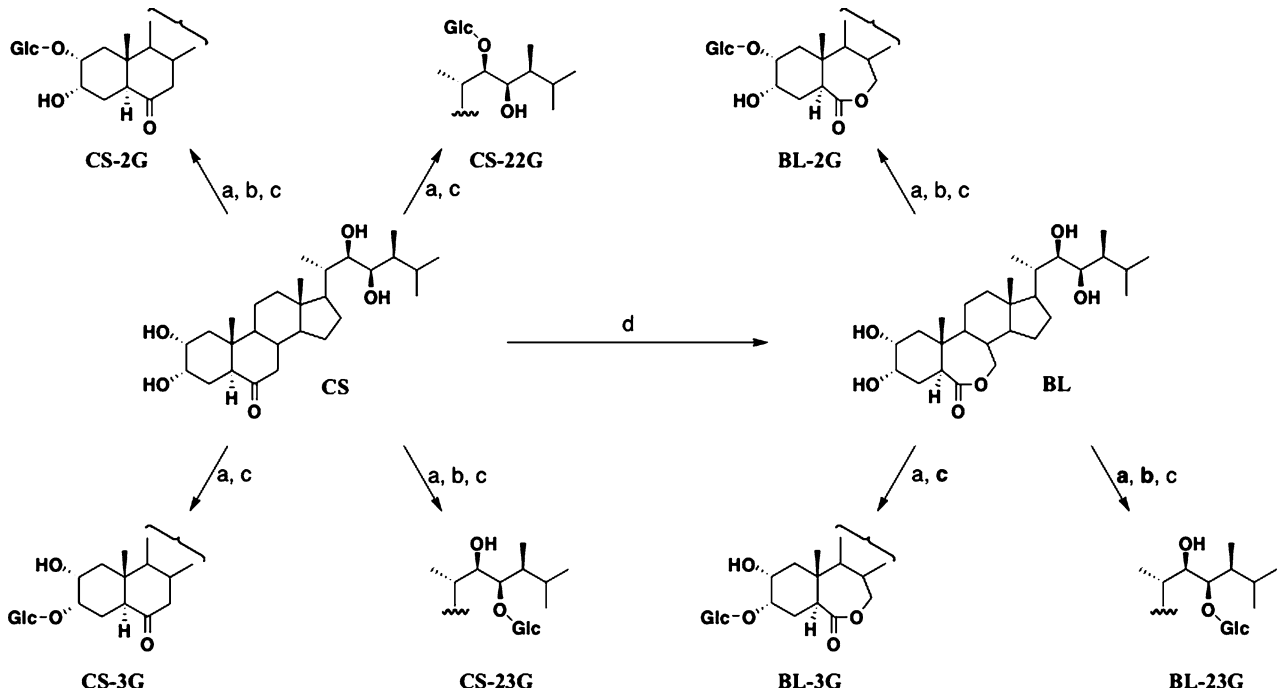

Figure 2. Glucosylation profiles of castasterone (CS) and BL in (a) mung bean explants, (b) *A. thaliana* seedlings, and (c) cultured *Catharanthus roseus* cells. Bold letters indicate the main glucosylation pathway in each plant species. (d) This conversion was demonstrated in *A. thaliana* (Noguchi and others 2000) and *C. roseus* (Yokota and others 1990; Suzuki and others 1993a).

MS. No glucoside was detected in the control treatment. On feeding plants CS and BL, their 2-*O*- and 23-*O*-glucosides were identified as metabolites (Tables 2 and 3). No BR glucoside was detected in BR-treated medium. Therefore, seedlings do not appear to release metabolic glucosides into the medium. In *A. thaliana*, BL-23G was the major BR glucoside, as in mung bean. However, its conversion rate was much lower than in the mung bean, and glucosylation occurred only at the C-2 and C-23 hydroxyl groups of CS and BL.

To investigate whether the pattern of BL-23G accumulation in *A. thaliana* depends on incubation time, time course experiments were carried out. The results are summarized in Figure 1. After a 1-day incubation, BL-23G was detected, and its levels increased significantly after a 2-day incubation. After a 3-day incubation, however, the level decreased, whereas the fresh weight (fw) of *A. thaliana* continued to increase. Therefore, BL-23G appears to be transient in the BL inactivation cascade in *A. thaliana*, and it is probably further metabolized, perhaps by conjugation. In tomato cell suspension cultures, 24-*epi*-teasterone was conjugated into its 3-glucoside and 3-disaccharide (Kolbe and others 1997, 1998). Recently, UDP-glycosyltransferases (UGTs) of *A. thaliana*, which catalyze 23-*O*-glucosylation of the BL and CS, were identified, and transgenic plants overexpressing that UGTs displayed BR-deficient phenotypes and contained reduced amounts of BRs (Poppenberger and others 2005). These results suggest that glucosylation of BRs regulates BR activity in *Arabidopsis*.

Metabolism of Castasterone and Brassinolide in Cultured **C. roseus** *Cells.* The metabolism of CS and BL was also investigated using cultured *C. roseus* cells. After 1-, 2-, 3-, or 7-day incubations, their metabolites were purified and analyzed using LC/MS/MS. The glucosylation profile after the 7-day incubation is summarized in Tables 2 and 3. In cultured *C. roseus* cells, CS and BL were converted into several glucosides and they were glucosylated at the same hydroxyl groups identified in the mung bean experiments. However, cultured *C. roseus* cells seem to prefer to glucosylate CS and BL at C-3 over other positions, and BL-3G was the main glucoside in *C. roseus*. After a 1-day incubation, BL-3G was detected, and the amount increased gradually for 7 days (data not shown); nevertheless, even after a 7-day incubation, the conversion rate was not large (Table 2) compared with that of BL-23G in mung bean and *A. thaliana*. Therefore, the glucosylation of CS and BL appears limited in cultured *C. roseus* cells.

Conclusions

The glucosylation profiles of CS and BL are summarized in Figure 2. This study provides the first evidence for the metabolic conversion of CS into CS-2G, CS-3G, CS-22G, and CS-23G, and the conversion of BL into BL-2G and BL-3G, and it expands our knowledge of the glucosylation of BRs. In all three plant species examined, the same four BR glucosides were identified (CS-2G, CS-23G, BL-2G, and BL-23G), although the glucosylation profiles varied with plant species. In addition, the glucosylation of CS appeared limited (Yokota and others 1987; Soeno and others 2000), compared with that of BL. So far, the isolation and identification of endogenous glycosides has been very limited. Although we clearly demonstrated their metabolic conversion, endogenous glucosides were not identified in any plant species examined, suggesting that the levels of endogenous BR glucosides are very low. Therefore, the development of a more sophisticated method for detecting BR glucosides is necessary to gain knowledge of endogenous BR glucosides.

REFERENCES

Clouse SD. 2002. Brassinosteroids—plant counterparts to animal steroid hormones? Vitamins Hormones 65:195–223.

Fujioka S, Sakurai A. 1997. Brassinosteroids. Natural Product Rep 14:1–10.

Fujioka S, Yokota T. 2003. Biosynthesis and metabolism of brassinosteroids. Annu Rev Plant Biol 54:137–164.

Hai T, Schneider B, Adam G. 1995. Metabolic conversion of 24-*epi*-brassinolide into pentahydroxylated brassinosteroid glucosides in tomato cell cultures. Phytochemistry 40:443–448.

Hai T, Schneider B, Porzel A, Adam G. 1996. Metabolism of 24-*epi*-castasterone in cell suspension cultures of *Lycopersicon esculentum*. Phytochemistry 41:197–201.

Kolbe A, Porzel A, Schneider B, Adam G. 1997. Diglycosidic metabolites of 24-*epi*-teasterone in cell suspension cultures of *Lycopersicon esculentum* L. Phytochemistry 46:1019–1022.

Kolbe A, Schneider B, Porzel A, Adam G. 1998. Metabolic inversion of the 3-hydroxy function of brassinosteroids. Phytochemistry 48:467–470.

Noguchi T, Fujioka S, Choe S, Takatsuto S, Tax FE, and others. 2000. Biosynthetic pathways of brassinolide in *Arabidopsis*. Plant Physiol 124:201–209.

Poppenberger B, Fujioka S, Soeno K, George GL, Vaistij FE, and others. 2005. The UGT73C5 of *Arabidopsis thaliana* glucosylates brassinosteroids. Proc Natl Acad Sci USA 102:15253–15258.

Schneider B, Kolbe A, Porzel A, Adam G. 1994. A metabolite of 24-*epi*-brassinolide in cell suspension cultures of *Lycopersicon esculentum*. Phytochemistry 36:319–321.

Soeno K, Kyokawa Y, Natsume M, Abe H. 2000. Teasterone-3-*O*-*β*-D-glucopyranoside, a new conjugated brassinosteroid metabolite from lily cell suspension cultures and its identification in lily anthers. Biosci Biotechnol Biochem 64:702–709.

Suzuki H, Fujioka S, Takatsuto S, Yokota T, Murofushi N, and others. 1993a. Biosynthesis of brassinolide from castasterone in culture cells of *Catharanthus roseus*. J Plant Growth Regul 12:101–106.

Suzuki H, Kim SK, Takahashi N, Yokota T. 1993b. Metabolism of castasterone and brassinolide in mung bean explant. Phytochemistry 33:1361–1367.

Yokota T. 1999. Brassinosteroids In: Hooykaas PJJ, Hall MA, Libbenga KR, editors. Biochemistry and Molecular Biology of Plant Hormones. Elsevier Science: Amsterdam, the Netherlands, pp 277–293.

Yokota T, Kim SK, Kosaka Y, Ogino Y, Takahashi N. 1987. Conjugation of brassinosteroids. In: Schreiber K, Schütte HR, Sembdner G, editors. Proceedings of the International Symposium. Berlin, Germany, VEB Deutscher Verlag der Wissenschaften, pp 288–296.

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