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BRASSINOSTEROIDS IN *ARABIDOPSIS THALIANA*

SHOZO FUJIOKA,†* TAKAHIRO NOGUCHI,†‡ TAKAO YOKOTA,§ SUGURU TAKATSUTO¶ and SHIGEO YOSHIDA†

† The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-01, Japan; ‡ Tama Biochemical Co., Ltd., Shinjuku-ku, Tokyo 163, Japan; § Department of Biosciences, Teikyo University, Utsunomiya 320, Japan; ¶ Department of Chemistry, Joetsu University of Education, Joetsu-shi, Niigata 943, Japan

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Abstract—From the seeds and siliques of *Arabidopsis thaliana*, six brassinosteroids, brassinolide, castasterone, typhasterol, 6-deoxocastasterone, 6-deoxotyphasterol and 6-deoxoteasterone, were identified by GC-mass spectrometry or GC-selected ion monitoring. As the occurrence of castasterone, typhasterol, 6-deoxocastasterone and 6-deoxotyphasterol in the shoots of *A. thaliana* has already been reported, this study provides evidence for the occurrence of the above four brassinosteroids in different organs, seeds and siliques, and the first evidence for the occurrence of brassinolide and 6-deoxoteasterone in *A. thaliana*. All brassinosteroids identified in this study belong to important components of both the early and late C-6 oxidation pathways, which were established in the cultured cells of *Catharanthus roseus*. This suggests that both pathways are operating in *A. thaliana* to produce the most biologically active brassinosteroid, brassinolide, which is responsible for growth and development of the plant.

INTRODUCTION

Brassinosteroids (BRs) are an important class of plant hormones, which play essential roles for plant growth and development [1–6] although this has not received acceptance until recently. However, the establishment of biosynthetic pathways of BRs in *Catharanthus roseus* [1–3, 6], the discovery of BR biosynthetic mutants and BR insensitive mutants of *Arabidopsis thaliana* [7–10] and *Pisum sativum* [11] has led now to wide acceptance of this view. Although the basic information of endogenous BRs in *Arabidopsis* is a prerequisite for the examination of BR biosynthetic pathways and BR mutants in *Arabidopsis*, little information has been available. Recently Fujioka *et al.* [12] provided the first evidence for the occurrence of castasterone (**2**), typhasterol (**3**), 6-deoxocastasterone (**4**) and 6-deoxotyphasterol (**5**) by GC-MS in the shoots of the wild type *Arabidopsis* (Columbia). Subsequently, Schmidt *et al.* [13] reported that seeds of *Arabidopsis* (ecotype 24) contain 24-epibrassinolide and **2**. In this study, we have further investigated the natural occurrence of BRs in several lots of seeds and siliques of the wild type *Arabidopsis* (Columbia) to obtain more information on the biosynthesis of the endogenous BRs in *Arabidopsis*.

* Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Two lots of mature seeds were analyzed for endogenous BRs. In the first experiment, mature seeds (5 g fr. wt) of the wild type *Arabidopsis* (Columbia) were extracted with methanol–chloroform. After concentration of the combined extracts *in vacuo* the residue was partitioned between water and chloroform. The chloroform extract was purified by silica gel column chromatography, Sephadex LH-20 column chromatography and separation with an ODS cartridge. A BR-enriched fraction was further purified by ODS-HPLC, and appropriate fractions were derivatized and analyzed by GC-MS or GC-SIM. Based on the full mass spectrum and GC retention time, compound **2** was identified (Table 1). Brassinolide (**1**), **4**, **5** and **6** were identified by GC-SIM (Table 1). Their approximate amounts were estimated based on the GC-MS or GC-SIM data. In this lot of seeds, compound **2** (ca 5 ng g^{-1} fr. wt) and **4** (ca 1.5 ng g^{-1} fr. wt) were relatively abundant, and the amounts of **1**, **5** and **6** were ca 0.5–1 ng g^{-1} fr. wt.

In the second experiment, a different batch of mature seeds (10 g fr. wt) of the wild type *Arabidopsis* (Columbia) were extracted with methanol–chloroform. In this experiment, deuterium labeled internal standards were added to the extract to quantify BRs. The purification was carried out according to the same method as the case of the first experiment. HPLC-purified fractions were derivatized and analyzed by

Table 1. Identification of brassinosteroids in the seeds of *Arabidopsis thaliana*

Compound	R_t in GC	Characteristic ions *m/z* (relative intensity %)
Brassinolide (**1**)[a]	12′30″	528 [M^+] (5), 374 (29), 177 (77), 155 (100)[b]
Castasterone (**2**)[c]	11′40″	512 [M^+] (73), 399 (16), 358 (24), 287 (35), 155 (100)
Typhasterol (**3**)[a,d]	11′01″	544 [M^+] (100), 529 (60)[b]
6-Deoxocastasterone (**4**)[a]	10′38″	498 [M^+] (18), 273 (100), 155 (17)[b]
6-Deoxotyphasterol (**5**)[a]	10′06″	530 [M^+] (20), 440 (17), 215 (100)[b]
6-Deoxoteasterone (**6**)[a]	10′30″	530 [M^+] (45), 440 (14), 215 (100)[b]

[a] Identified by GC-SIM.
[b] Monitored ions by GC-SIM.
[c] Identified by full scan GC-MS.
Compounds **1**, **2** and **4** were converted to bismethaneboronates.
Compounds **3**, **5** and **6** were converted to methaneboronate-trimethylsilyl ethers.
[d] Typhasterol was identified in the second experiment using a different lot of seeds, in which **1**, **2**, **4**, **5** and **6** were also identified by GC-SIM.

GC-SIM. In this experiment, six BRs (**1**, **2**, **3**, **4**, **5**, **6**) were identified and the quantitative data are summarized in Fig. 1. In this lot of seeds, compounds **1**, **3** and **4** were relatively abundant.

As siliques are also expected to be one of the richest source of BRs in plant organs, we have examined endogenous BRs using two different lots of siliques. Extraction and purification were conducted according to the method described in the experimental section. In 6 g fr. wt of siliques collected from the plants grown in a growth chamber, compound **2** was identified by full-scan GC-MS (**2**-bismethaneboronate (BMB); *m/z* (rel. int.) 512 [M^+] (66), 441 (8), 399 (15), 358 (22), 287 (31), 155 (100)). Compounds **1** and **4** were identified by GC-SIM with monitoring ions at *m/z* 528 [M^+], 332, and 155 characteristic of **1**–BMB, and at *m/z* 498 [M^+], 273 and 155 characteristic of **4**-BMB. Among the detected BRs, compound **2** was the most abundant, and its amount was estimated to be 4 ng g^{-1} fr. wt.

Among 30 g fr. wt of siliques collected from the plants grown in a growth room, two thirds (20 g fr. wt equivalent) were analyzed for the presence of BRs. Compounds **4** and **5** were identified by full-scan GC-MS (**4**-BMB; *m/z* 498 [M^+] (24), 483 (8), 278 (100), 205 (34), 155 (49); **5**-methaneboronate-trimethylsilyl ether (MB-TMSi): *m/z* 530 [M^+] (11), 515 (4), 440 (15), 425 (25), 215 (100), 155 (14)). Using one third (10 g fr. wt equivalent) of the same lot of siliques, endogenous BRs were quantified by GC-SIM using internal standards. In addition to **4** and **5**, compound **3** was identified from this experiment. Due to impurities, the occurrence of **1** and **2** was not confirmed. The concentrations of **5** (5.4 ng g^{-1} fr. wt) and **4** (3.0 ng g^{-1} fr. wt) were high, while a lower amount of **3** (1.3 ng g^{-1} fr. wt) was observed.

In the present study, we have analyzed four different samples of seeds and siliques (two lots each). Altogether, six BRs (**1**, **2**, **3**, **4**, **5** and **6**) were identified from these samples. This study demonstrated the occurrence of **1** and **6** in *Arabidopsis* for the first time. Although Schmidt *et al.* [13] reported the occurrence of 24-epibrassinolide in seeds, we could not detect this BR in all samples of seeds and siliques analyzed in this study. Instead of 24-epibrassinolide, compound **1** was detected in both seeds and siliques. Very recently, we also found **1** in the shoots of *Arabidopsis* (S. Fujioka *et al.*, unpublished results). Therefore, compound **1**, the most biologically active BR, could be responsible for plant growth and development of *Arabidopsis*. The BRs identified in this study include the biosynthetic intermediates which belong to the early C-6 oxidation and late C-6 oxidation pathways which were established in cultured cells of *Catharanthus roseus* [3, 6, 14, 15]. Therefore, together with our previous study [12, 16], the present study strongly suggests that both biosynthetic pathways are operating in *Arabidopsis*. Endogenous amounts of each BR were rather different in each of two lots of seeds and siliques. Thus, pool levels of endogenous BRs seem to be variable depending on the developmental stages or growth conditions. More detailed analysis would clarify any fluctuation of endogenous BRs according to the development of the silique or stage of seed maturation.

EXPERIMENTAL

GC-MS analysis

The GC-MS analysis was carried out under the following conditions: Automass (JMS-AM150) mass spectrometer connected with a Hewlett-Packard 5890A-II gas chromatograph, EI (70 eV), source temp. 210°, column DB-5 (J&W, 15 m × 0.25 mm, 0.25 μm film thickness), injection temp. 250°, column temp. programme: 80° for 1 min, then raised to 320° at a rate of 30° min^{-1} and held on this temperature for 5 min; interface temp. 250°, carrier gas He, flow rate 1 ml min^{-1}, splitless injection. The samples corresponding to **1**, **2** and **4** were treated with pyridine containing methaneboronic acid (2 mg ml^{-1}), and the

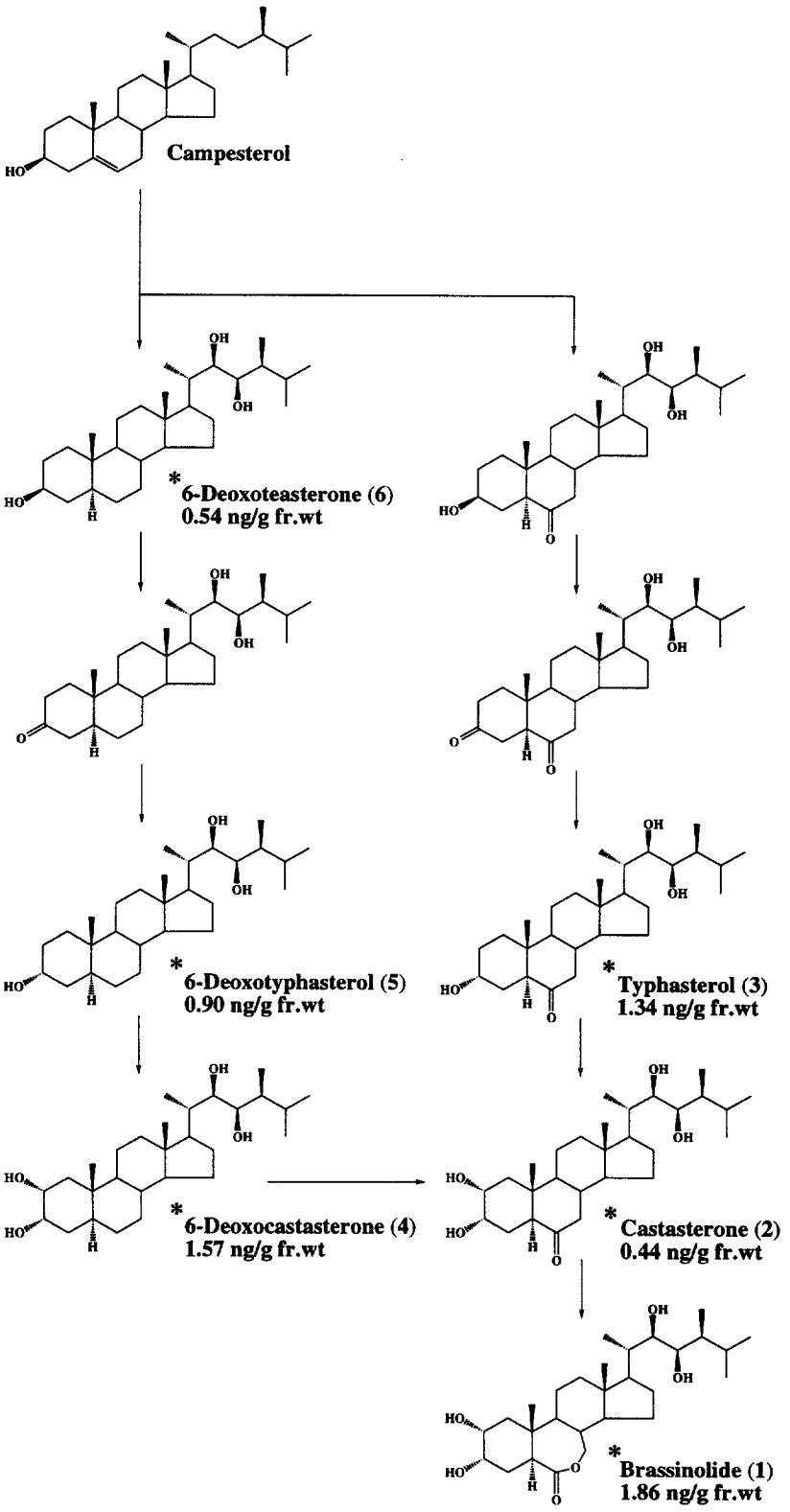

Fig. 1. Putative biosynthetic pathway of brassinolide in *Arabidopsis thaliana*. Asterisked brassinosteroids were identified in this study. The values (ng g^{-1} fr. wt) show endogenous amounts obtained by analysis of the seeds (second lot) of the wild type *Arabidopsis thaliana*.

samples corresponding to **3**, **5** and **6** were treated with pyridine containing methaneboronic acid (2 mg ml^{-1}), then with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide.

Plant material

Columbia (Col-0) wild type *Arabidopsis* seeds were purchased from LEHLE SEEDS (Round Rock, TX, U.S.A.). First lot of seeds (5 g fr. wt): Seed Lot No. GH196-13 (1.0 g fr. wt × 5), and second lot of seeds (10 g fr. wt): Seed Lot No. GH197-06 (1.0 g fr. wt × 10).

Six g fr. wt of siliques were collected from *Arabidopsis* wild type (Columbia) plants which were grown in a growth chamber at 22° under continuous light (ca 4000 lux). A second batch of siliques (30 g fr. wt) was collected from *Arabidopsis* wild type (Columbia) plants which were grown in a growth room at 25° under continuous light (ca 3000 lux).

Extraction and purification

The first lot of seeds (5 g fr. wt) were extracted 3 × with MeOH–$CHCl_3$ (4:1, 100 ml). The combined extracts were evapd to dryness *in vacuo*. The residue was partitioned 3 × between H_2O (30 ml) and $CHCl_3$ (30 ml). The $CHCl_3$ fr. was chromatographed on a silica gel column (Wakogel C300, 10 g). Elution was carried out with $CHCl_3$ (100 ml) and $CHCl_3$–MeOH (9:1, 100 ml). The eluate with 10% MeOH was subjected to a Sephadex LH-20 column (column vol. 200 ml). Elution was carried out with MeOH–$CHCl_3$ (4:1) to give BRs fraction (Ve/Vt: 0.6–0.8). This fr. was charged onto a C18 cartridge (Sep-Pak Plus, Waters) and eluted with 20 ml of MeOH. The fr. was further purified by ODS-HPLC (Senshu Pak, ODS-1151-D, 4.6 × 150 mm); flow rate 0.8 ml min^{-1}, mobile phase, MeCN–H_2O (45% MeCN for 15 min, then raised to 80% MeCN within 3 min and held on 80% MeCN for 12 min, then raised to 100% MeCN within 2 min and held on 100% MeCN). Frs were collected every 2.5 min (R_t 7.5–50 min), concd, derivatized, and examined by GC-MS or GC-SIM. The main frs of **1**, **2**, **4**, **6** and **5** were R_t 10–12.5 min, 12.5–15 min, 30–32.5 min, 35–37.5 min and 40–42.5 min, respectively.

For the quantitative analysis, 10 g fr. wt of the seeds were extracted 3 × with MeOH–$CHCl_3$ (4:1, 150 ml). Internal standards (100 ng of each deuterium-labeled BRs: [$^2H_6$]**1**, [$^2H_6$]**2**, [$^2H_6$]**3**, [$^2H_6$]**4**, [$^2H_6$]**5** and [$^2H_6$]**6** [14, 15, 17]) were added to the extract. The purification was carried out as described above. The endogenous levels of BRs were determined as the ratio of the peak areas of molecular ions for the endogenous one and for the internal standard. The [$^2H_0$] and [$^2H_6$] ions monitored to quantify individual BRs were as follows: *m/z* 528 and 534 (M^+ of **1**-BMB); *m/z* 512 and 518 (M^+ of **2**-BMB); *m/z* 544 and 550 (M^+ of **3**-MB-TMSi); *m/z* 498 and 504 (M^+ of **4**-BMB); *m/z* 530 and 536 (M^+ of **5** and **6**-MB-TMSi).

The first lot of siliques (6 g fr. wt) were extracted 3 × with MeOH (50 ml). After evaporation of MeOH, the residue was partitioned 3 × between H_2O (20 ml) and $CHCl_3$ (20 ml). The $CHCl_3$ fr. was subjected to silica gel cartridge (Sep-Pak Vac 12 cc (2 g), Waters). The column was eluted with 20 ml of $CHCl_3$, followed by 2% MeOH in $CHCl_3$ and 7% MeOH in $CHCl_3$. Each of the 2% MeOH and 7% MeOH was prepurified by C18 cartridge (Sep-Pak Plus, Waters) with MeOH, and each eluate was subjected to ODS-HPLC (Senshu Pak, ODS-1151-D, 4.6 × 150 mm). Frs were collected every 2.5 min (R_t 7.5–50 min), concd, derivatized and examined by GC-MS or GC-SIM. From HPLC frs derived from the 7% MeOH fr., **1** (R_t 10–12.5 min), **2** (12.5–15 min) and **4** (R_t 30–32.5 min) were identified.

Two thirds of a second lot of siliques (20 g fr. wt) were extracted 3 × with MeOH (100 ml). The combined extract was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ fr. was subjected to silica gel CC (Wako gel C300, 10 g). The column was eluted with 100 ml of $CHCl_3$, followed by 2% MeOH in $CHCl_3$ and 7% MeOH in $CHCl_3$. Each of the 2% MeOH and 7% MeOH frs was purified by Sephadex LH-20 (column vol. 200 ml, MeOH:$CHCl_3$ = 4:1), and the effluents of Ve/Vt: 0.6–0.8 were collected. After purification by C18 cartridge column with MeOH, the eluates were subjected to ODS-HPLC (Senshu Pak ODS-4053-N; 10 × 50 mm + ODS-5251-N; 20 × 250 mm, Senshu Scientific Co., Ltd.) at a flow rate of 8 ml min^{-1} with the solvents of 65% MeCN for the eluate derived from the 7% MeOH fr. and of 80% MeCN for the eluate derived from the 2% MeOH fr. Frs were collected every 5 min (R_t 5–70 min), concd, derivatized, and examined by GC-MS. Compound **4** was identified from HPLC fr. (R_t 40–45 min) derived from the 7% MeOH fr. Compound **5** was identified from HPLC fr. (R_t 55–60 min) derived from the 2% MeOH fr.

For the quantitative analysis, 10 g fr. wt of the siliques (1/3 of the second lot) were extracted 3 × with MeOH (50 ml). Internal standards (100 ng of each deuterium-labeled BRs: [$^2H_6$]**1**, [$^2H_6$]**2**, [$^2H_6$]**3**, [$^2H_6$]**4**, [$^2H_6$]**5** and [$^2H_6$]**6**) were added to the extract. The purification was carried out according to the same method as the case of seeds.

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