6-Deoxocastasterone and Its Biosynthetic Precursors from Primary Roots of Maize

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Brassinosteroids (BRs) are the collective name for a group of steroidal plant hormones which are essential chemical signals for the growth and normal development of plants.[1,2] Up to date, over forty different members of the BR group have been identified from various plant materials.[3,4] Among them, brassinolide (BL) and castasterone (CS) are considered to be the most important BRs, due to both their ubiquity in the plant kingdom, and their strong biological activity.[1-3] For this reason, biosynthesis of these BRs has been extensively investigated, usually by feeding experiments using isotope-labeled substrates, and molecular genetic analyses of BR-deficient mutants. These studies have demonstrated that CS is biosynthesized from campesterol (CR) via two parallel pathways, namely the early and late C6-oxidation pathway, and then CS synthesizes BL by 7-oxalactonation.[5,6]

Recently the presence of a BR, CS, was the first to be demonstrated in the primary roots of maize.[7] In addition, BRs applied exogenously to the maize roots activated growth and gravitropic curvature of the roots, suggesting that BRs play important roles in the growth and development of plant roots.[7] Compared to the wealth of data regarding BR biosynthesis in the aerial parts of plants, however, little is known about BR biosynthesis in the underground portions of plant, namely, the roots.[3,4] This led us to re-investigate endogenous BRs, and their biosynthetic precursors, in the maize primary roots. These experiments were on a larger scale than previous experiments which helped us understand how BRs are biosynthesized in the roots.

Primary roots of maize were extracted with 80% methanol, followed by chloroform. For facile purifications and quantitative analyses, deuterium-labeled ([26,28-$^2H_6$]) BRs which are biosynthetically related to CS, such as [$^2H_6$]-6-deoxoCS, -teasterone (TE), -typhasterol (TY) and -brassinolide (BL), were added to chloroform-soluble extract (Figure 1). The extract was dried *in vacuo*, and partitioned between *n*-hexane and 80% methanol. BRs in the 80% methanol fraction were purified by column chromatography, and subjected to reversed-phase HPLC. The HPLC fractions were collected every minute, and biological activity in the fractions was determined by the rice lamina inclination assay.[8]

As shown in Figure 2, BR activity was detected in fractions 11-12, 16, 31, 38-39 and 46. Under the same HPLC conditions, authentic [$^2H_6$]-BL, -CS, -TE, -TY and -6-

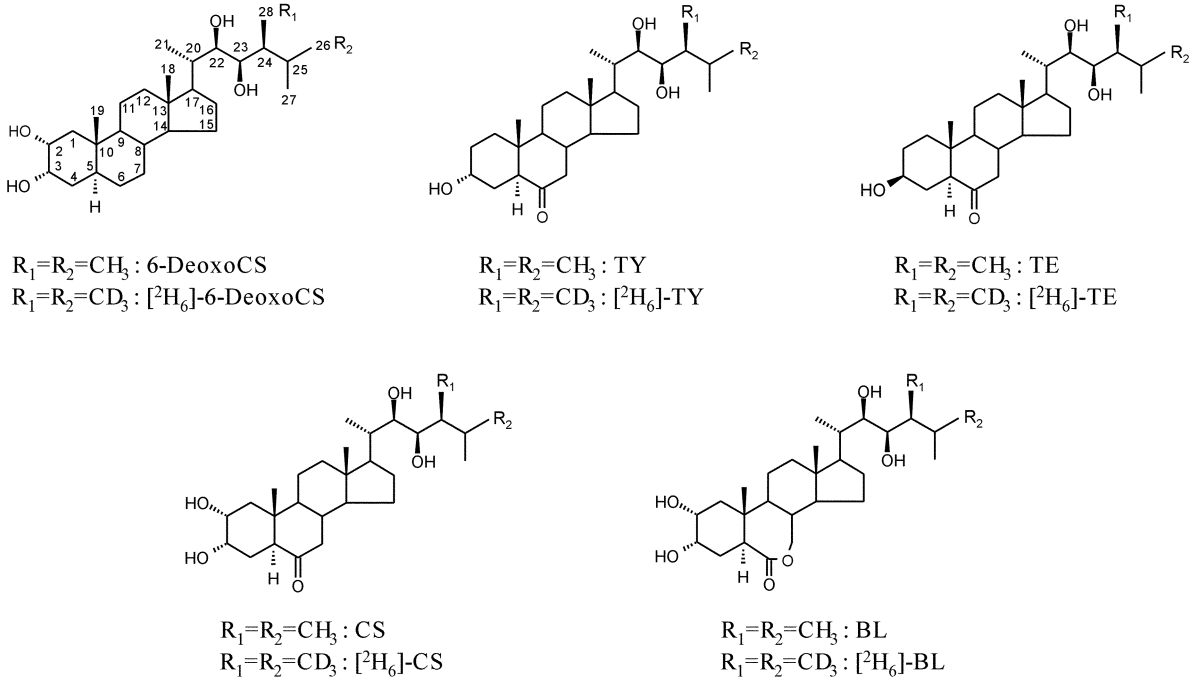

Figure 1. Structure of BRs.

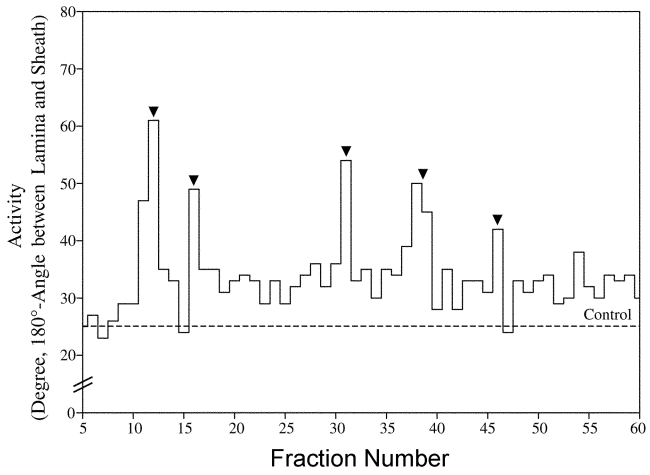

Figure 2. Distribution of biological activity of BRs in primary roots of maize, determined by rice lamina inclination assay after reversed-phase HPLC.

deoxoCS were eluted in the fractions 11-12, 16, 31, 38-39 and 46, respectively, indicating that the fractions contained the [$^2H_6$]-BRs which was added as an internal standard. Therefore, the presence of endogenous non-labeled ([$^2H_0$]) BL, CS, TE, TY and 6-deoxoCS in fractions 11-12, 16, 31, 38-39, 46, respectively, was examined by GC-MS and GC-selected ion monitoring (SIM) after suitable derivatization. Bismethaneboronate (BMB) of an active compound in fraction 16 gave a molecular at *m/z* 512 and prominent ions at *m/z* 155, 287, 327, 358, 399 and 497. The mass spectrum and GC retention times were identical to those derived from authentic CS BMB (Table 1), demonstrating that the isolated compound was, indeed, CS. Endogenous amounts of CS in the roots was calculated by the ratio of CS/[$^2H_6$]-CS, which was added as an internal standard, and was approximately 0.9 ng g fr.wt^{-1}. In fraction 46, the BMB of an active compound showed characteristic ions for 6-deoxoCS BMB at *m/z* 498, 483, 332, 273, 213 and 155, whose retention time on GC was the same as that of synthetic 6-deoxoCS BMB. Therefore, the compound was determined to be 6-deoxoCS. The amount of 6-deoxoCS in the roots was approximately 0.3 ng g fr.wt^{-1} on the basis of relative intensity, against [$^2H_6$]-6-deoxoCS, added to the fraction as an internal standard. In contrast, GC-MS/SIM analysis revealed that fractions 11-12, 31, and 38-39 contained [$^2H_6$]-BL, -TE and -TY, respectively, but non-labeled endogenous BL, TE and TY were not detected in the fractions (data not shown). In consequence, along with the previously-identified CS,[5] 6-deoxoCS was newly identified in the primary roots of maize.

6-DeoxoCS and CS are known be biosynthesized from CR *via* campestanol (CN) by the late C6-oxidation pathway.[3,4] Therefore, the presence of CR and CN as biosynthetic precursors of BRs in the maize roots was expected. To verify this, 4-demethylsterols in the hexane-soluble fraction obtained from the maize roots were acetylated, and analyzed by capillary GC-MS. As summarized in Table 2, two endogenous 4-demethylsteryl acetates were detected at the same GC retention times (15.17 and 15.42 min) as that of authentic campesteryl (15.17 min), and also campestanyl acetate (15.42 min). Furthermore, the obtained mass spectra of the 4-demethylsteryl acetates were identical to those of campesteryl and campestanyl acetate, clearly suggesting that the maize roots contained CR and CN.

Our previous study revealed that maize shoots, aerial parts of seedlings, also contain 6-deoxoCS and CS.[9] Although the presence of biosynthetic precursors of endogenous BRs, CR, and CN was demonstrated in the maize roots, the possibility that 6-deoxoCS and CS had been transported to the roots after being synthesized in the shoots was not excluded. To ascertain the biological origin of BRs in the roots, *e.g.* whether it had been transported from the shoots or biosynthesized in the roots, the presence of 6-deoxoCS oxidase, an enzyme catalyzing the conversion of 6-deoxoCS to CS in the maize root, was examined. This was accomplished using a crude enzyme solution prepared from the maize roots. After confirming that the endogenous amount of the expected product, CS, was negligible (data not

Table 1. GC-MS data for autherntic and endogenous CS and 6-deoxoCS in primary roots of maize

Compound*	Rt** (min) on GC	Prominent ions (*m/z*, relative intensity %)
Endogenous CS	21.96	512 (M^+, 82), 497 (5), 399 (23), 358 (40), 327 (9), 287 (48), 155 (100)
Endogenous 6-deoxoCS	15.49	498 (M^+, 34), 483 (15), 332 (5), 273 (100), 213 (4), 155 (35)
Authentic CS	21.96	512 (M^+, 81), 497 (5), 399 (22), 358 (38), 327 (8), 287 (46), 155 (100)
Authentic 6-deoxoCS	15.49	498 (M^+, 35), 483 (15), 332 (4), 273 (100), 213 (4), 155 (37)

*Compound was analyzed as a bismethaneboronate derivative. **Rt: Retention time.

Table 2. GC-MS data for authentic and endogenous campesterol (CR) and campestanol (CN) in maize primary roots

Compound*	Rt** (min) on GC	Prominent ions (*m/z*, relative inetnsity %)
Authentic CR	15.17	442 (M^+, trace), 382 (100), 367 (22), 274 (12), 255 (14), 213 (10), 159 (13)
Authentic CN	15.42	444 (M^+, 39), 384 (61), 369 (53), 275 (37), 257 (20), 230 (24), 215 (100), 161 (16)
Endogenous CR	15.17	442 (M^+, trace), 382 (100), 367 (21), 274 (13), 255 (14), 213 (10), 159 (13)
Endogenous CN	15.42	444 (M^+, 38), 384 (62), 369 (54), 275 (37), 257 (20), 230 (23), 215 (100), 161 (15)

*Compound was analyzed as a derivative of acetate. **Rt: Retention time

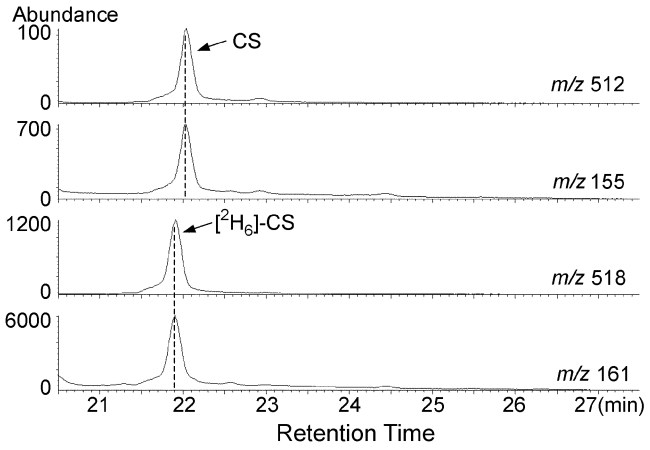

Figure 3. GC-SIM analysis of the 6-deoxoCS oxidase product, prepared from primary roots of maize.

shown) in the enzyme solution, 6-deoxoCS was added to the solution, as a substrate. The assay mixture was incubated at 37 ºC for 30 minutes, [$^2H_6$]-CS was added as an internal standard for quantitative analysis, and the mixture was extracted with ethyl acetate. The ethyl acetate-soluble fraction was purified by reversed-phase HPLC, and the HPLC fraction corresponding to CS was analyzed by GC-SIM, after methaneboronation. The BMB of the product exhibited a molecular peak at *m/z* 512, and a base peak at *m/z* 155, with a GC retention time equal to that of authentic CS BMB. The specific activity of 6-deoxoCS oxidase, measured by the ratio of the product to the internal standard, was approximately 0.05 ng mg protein^{-1} min^{-1} (Figure 3). Therefore, the presence of 6-deoxoCS oxidase was verified in the maize roots.

6-DeoxoCS is a direct biosynthetic precursor of CS. The presence of 6-deoxoCS and CS in primary roots of maize suggests that CS is biosynthesized from 6-deoxoCS in the roots. Actually, the maize roots contain biosynthetic BR precursors (CR and CN), and an enzyme mediating the conversion of 6-deoxoCS to CS, 6-deoxoCS oxidase. This also suggests that the maize roots are capable of synthesizing BRs. Nevertheless, the bio-origin of BRs in maize roots is still controversial, because maize seedling shoots and germinating seeds also contain 6-deoxoCS and CS,[9] which can be transported to the roots to control growth and differentiation during the early germination stage. To further determine BR origin in the roots, the possible activity of enzymes catalyzing reactions indicative of the synthesis of 6-deoxoCS from CR is now underway.

6-DeoxoCS is a member of the late C6-oxidation pathway, whereas TE and TY are members of the early C6-oxidation pathway. Suzuki *et al*. reported that maize pollen contains TE, TY and CS,[10] suggesting that BRs in the pollen are biosynthesized largely via the early C6-oxidation pathway. Coupled with CS, however, we identified 6-deoxoCS but not TE and TY, from the primary roots of maize. This denotes that the late C6-oxidation pathway is more active than the early C6-oxidation pathway in the production of BRs in maize roots. Taken together, it would appear that maize plants utilize both the early and late C6-oxidation pathway, but in a tissue-specific manner.

Experiment Section

Plant materials. Maize (*Zea mays* L cultivar Golden Cross Bantam) seeds were washed several times with tap water, and then soaked in distilled water for 24 hours. After soaking, the seeds were placed on trays (27 × 20 × 2.5 cm) covered by water-saturated paper towels. To keep the seeds moisturized, they were covered with one more layer of water-saturated paper towels. The trays were positioned vertically at 28 ºC in darkness, at 70% relative humidity. After germination in the dark for 2 days, 1.5-2 cm-long primary roots were collected from seedlings.

Purification of BRs in maize primary roots. Primary roots of maize (852 g) were extracted with 80% methanol (1 L × 3) followed by chloroform (1 L × 3). The chloroform-soluble fraction (3.8 g) was dried, and partitioned between *n*-hexane (1 L) and 80% methanol (1 L × 3). The 80% methanol phase was concentrated into an aqueous phase, and partitioned again between a phosphate buffer (0.1 M, pH 7.8) and ethyl acetate (1 L × 3). After drying, the ethyl acetate-soluble fraction was purified by silica gel column chromatography (Merck, 100 g), and eluted with increasing methanol content in chloroform (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50% methanol in chloroform). The rice lamina inclination bioassay showed BR activity in 4 and 5% methanol fractions. The fractions were combined, loaded onto a Sephadex LH-20 gel filtration column (22 × 900 mm), and eluted with a mixture of chloroform:methanol (4 : 1). The fractions which were eluted between an elution volume/total volume of between 0.65-0.75 were combined, and further purified by reversed-phase HPLC (8 × 100 mm, NovaPak, Waters) at a flow rate of 1 mL min^{-1} with 50% acetonitrile (MeCN)-water gradients: 0-25 minutes, 50% MeCN; 25-40 minutes, 50-100% MeCN; 40-70 minutes, 100% MeCN. The fractions eluted in the same retention time as that of authentic [$^2H_6$]-CS, -6-deoxoCS, -TE, -TY, and -BL were collected, then analyzed by capillary GC-MS/SIM after derivatization.

Purification of 4-demethylsterols in primary roots of maize. The dried residue (1.2 g) of the *n*-hexane soluble fraction was saponified with 70% ethanol (200 mL), containing 5% KOH, at 70 ºC for 90 minutes. The unsaponified lipids (800 mg) were extracted with *n*-hexane, and purified with a silica gel column (2.2 × 200 mm) using a mixture (1 : 1) of *n*-hexane and methylene chloride as an elution solvent. Based on the movement of a F_{254} preparatory TLC developed with ethanol free chloroform, 4-demethylsterols were separated from other lipids. The 4-methylsterols were acetylated with acetic anhydride and pyridine (v/v = 1 : 2) for 18 hours at room temperature. The 4-demethylsteryl acetates were adjusted to pH 7 with HCl solution (pH 3), and then extracted with *n*-hexane (200 mL × 3).

Assay for 6-deoxoCS oxidase in primary roots of

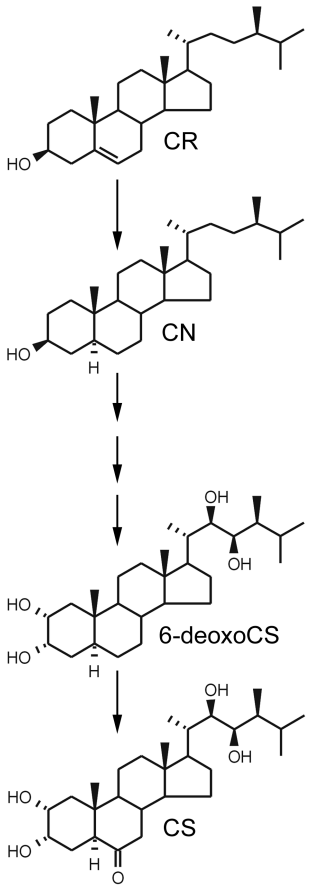

Figure 4. Proposed biosynthetic pathway in primary roots of maize.

maize. 6-deoxoCS oxidase activity was determined utilizing a cell-free crude enzyme solution obtained from the roots of maize. To this end, primary roots of maize (5 g) were homogenized with 0.1 M Na-phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 15 mM 2-mercaptoethanol, 15% glycerol, 250 mM sucrose, and 40 mM ascorbate. The homogenate was centrifuged at 15,000 × g for 30 minutes. The obtained supernatant was centrifuged again at 190,000 × g for 120 minutes. The resulting microsomal pellet was resuspended in 0.1 M Na-phosphate buffer (pH 7.4) containing 1.5 mM 2-mercapto-ethanol and 30% glycerol, and used as a crude enzyme solution for 6-deoxoCS oxidase.

Enzyme reaction was initiated by the addition of 6-deoxoCS as a substrate, in the presence of NADPH as a cofactor. After 30 minutes of incubation at 37 °C, [$^2H_6$]-CS was added to the reaction mixture as an internal standard for quantitative analysis, and the mixture was extracted with ethyl acetate (1.2 mL × 3). The ethyl acetate-soluble fraction was dried, dissolved in 50% methanol, and subjected to a SepPak (Waters, C_{18}) cartridge eluted with 50 and 100% methanol (5 mL × 2 each). The fraction eluted with 100% methanol was finally purified by reversed-phase HPLC, as described above. The fraction corresponding to CS in the same HPLC was collected and analyzed by GC-SIM, after methaneboronation.

GC-MS/SIM analysis. GC-MS/SIM analyses were carried out with a 5973 mass spectrometer (70 eV, Hewlett Packard) connected to a 6890 gas chromatograph, which was fitted with a fused silica capillary column (HP-5, 0.25 × 30 mm). GC conditions were as follows: on-column injection mode; He, 1 mL min^{-1}; oven temp, 175 °C for 2 minutes, thermal gradient from 175 °C to 280 °C at 40 °C min^{-1}, and kept 280 °C. Prior to injection, BRs was derivatized to be BMB or MB-TMSi ether.[11]

References

1. Clouse, S. D.; Sasse, J. *Annu. Rev. Plant Phsyiol. Plant Mol. Biol.* **1998**, *49*, 427.
2. Clouse, S. D.; Feldmann, K. A. *Brassinosteroids: Steroidal Plant Hormones*; Sakurai, A., Yokota, T., Clouse, S. D., Eds; Springer-Verlag: Tokyo, 1999; p 163.
3. Bajguz, A.; Tretyn, A. *Phytochemistry* **2003**, *62*, 1027.
4. Fujioka, S. *Brassinosteroids: Steroidal Plant Hormones*; Sakurai, A., Yokota, T., Clouse, S. D., Eds; Springer-Verlag: Tokyo, 1999; p 21.
5. Sakurai, A. In *Brassinosteroids: Steroidal Plant Hormones*; Sakurai, A., Yokota, T., Clouse, S. D., Eds.; Springer-Verlag: Tokyo, 1999; p 91.
6. Bishop, G. J.; Yokota, T. *Plant Cell Physiol.* **2001**, *42*, 114.
7. Kim, S.-K.; Chang, S. C.; Lee, E. J.; Chung, W.-S.; Kim, Y.-S.; Hwang, S. B.; Lee, J. S. *Plant Physiol.* **2000**, *123*, 997.
8. Arima, M.; Yokota, T.; Takahashi, N. *Phytochemistry* **1984**, *208*, 1.
9. Kang, M.-W.; Kim, Y.-S.; Kim, S.-K. *Korean J. Plant Biotech.* **2003**, *30*, 411.
10. Suzuki, H.; Yamaguchi, I.; Yokota, T.; Takahashi, N. *Agri. Biol. Chem.* **1986**, *50*, 3133.
11. Kim, T.-W.; Park, S.-H.; Joo, S.-H.; Kim, Y.-S.; Choo, J.; Kim, S.-K. *Bull. Korean Chem. Soc.* **2001**, *22*, 651.