Identification of Brassinosteroids and Their Biosynthetic Precursors from Seeds of Pumpkin

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Brassinosteroids are steroidal plant hormones which are essential for normal growth and differentiation of plants.[1~3] Recently, biosynthesis of brassinosteroids has been intensively investigated in not only higher plants[1~5] but also a lower plant.[6] Two common pathways for biosynthesis of brassinosteroids, namely the early- and late-C6 oxidation pathway, have been established. Although almost complete biosynthetic sequences for the pathways were determined by feeding experiments using isotope-labelled substrates and genetic analyses of brassinosteroids-deficient mutants, little information has been reported about enzymes involved in the biosynthetic pathways.[6,7]

A cell-free system is useful to investigate enzyme reactions in biosynthetic pathways of plant hormones. In researches for gibberellins biosynthesis, cell-free systems developed from endosperm of pumpkin seeds are the most widely used to examine enzymatic conversions, especially by hydroxylases.[8~11] In the views that brassinosteroids are polyhydroxysteroids and the hydroxylations are important to increase biological activity, cell-free systems developed from the seeds of pumpkin may be also useful to detect the enzyme reactions included in brassinosteroids biosynthesis. However, any attempt to develop cell-free systems for brassinosteroids biosynthesis in the seeds of pumpkin has not been carried out yet. To develop cell-free systems for brassinosteroids biosynthesis, thus, we preferentially investigated endogenous brassinosteroids and their biosynthetic precursors in seeds of pumpkin in this study. The results would provide valuable clues for biosynthetic pathway(s) of brassinosteroids in the seeds of pumpkin.

Seeds (3 kg) of pumpkin (*Cucurbita moschata*) were homogenized and extracted with 80% methanol (1 L × 3). The extracts were concentrated to the aqueous phase *in vacuo* and re-extracted with chloroform (1 L × 3). After reducing to dryness *in vacuo*, the chloroform soluble fraction was solvent-partitioned between *n*-hexane (1 L) and 80% methanol (1 L × 3). After drying, the 80% methanol soluble fraction was partitioned again between sodium phosphate buffer (pH 7.8) and ethyl acetate. Finally, *n*-hexane soluble fraction (250 g) containing phytosterols and ethyl acetate soluble fraction (7 g) containing brassinosteroids were obtained.

The ethyl acetate soluble fraction was purified by silica gel (100 g, Merck) column chromatography. The elution was carried out stepwise with chloroform containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 100% methanol (1 L each). In rice lamina inclination assay to detect brassinosteroids activity,[12] the 4-7% methanol in chloroform fractions showed biological activity. The fractions were combined and subjected to ODS (octadecylsilane, bed volume 100 mL, Merck LiChroprep RP-18) chromatography. The elution was carried out with the aqueous methanol increasing methanol content every 100 mL (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%). The biologically active fractions eluted with 80 and 90% methanol were combined and chromatographed on Sephadex LH-20 column (bed volume 340 mL: 22 × 90 mm) chromatography using a 4 : 1 mixture of methanol-chloroform at a flow rate 0.5 mL min^{-1}. The bioactive fractions with 0.65-0.75 of elution volume/total volume were combined, dissolved in small volume of methanol, and subjected to a reversed phase HPLC (8 × 100 mm, 4mm, Novapak C_{18} column) at a flow rate of 1 mL min^{-1} with 45% acetonitrile. Two biologically active peaks were detected in the bioassay and were referred to as fraction I and II based on their polarity (Figure 1).

The less polar fraction II was analyzed by a capillary GC-MS after methaneboronation.[13] Bismethaneboronate (BMB) of the active compound in fraction II gave molecular ion at m/z 512, and prominent ions at m/z 441, 399, 358, 327, 287 and 155, which are identical with those derived from authentic castasterone BMB. Moreover, GC retention of the active compound BMB was identical to that of authentic castasterone BMB detected in the same GC condition (Table 1). Thus, active compound in fraction II was determined to be castasterone (Figure 2).

In the same HPLC condition, authentic brassinolide is eluted in the fraction 7-8, suggesting that the fraction I was

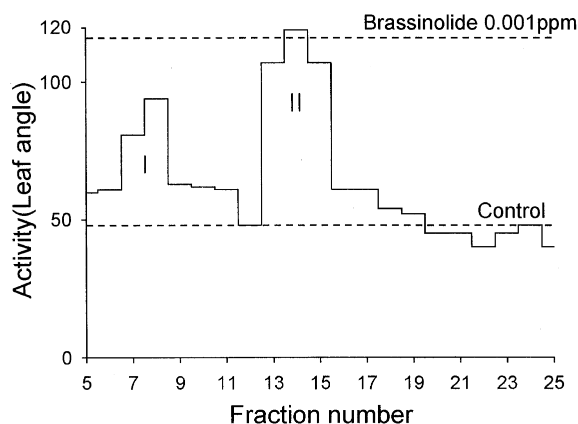

Figure 1. Separation of endogenous brassinosteroids in the seeds of pumpkin by a reversed phase HPLC. Extracts equivalent to 300 g fresh weight tissue were bioassayed by the rice lamina inclination test.

Table 1. Identification of brassinosteroids in *C. moschata* by GC-MS

Compound	Rt on GC (min)	Prominent ions (m/z, relative intensity)
Fraction I*	20.11	155(100), 177(79), 332(27), 374(13), 457(7), 528(5)
Fraction II**	16.47	155(100), 287(28), 327(8), 358(24), 399(10), 441(10), 512(58)
Brassinolide**	20.11	155(100), 177(73), 332(40), 374(31), 457(7), 528(5)
Castasterone**	16.47	155(100), 287(35), 327(10), 358(28), 399(13), 441(10), 512(73)

GC-MS (EI, 70 eV) was carried out by Hewlett-Packard 6890-5973 fitted with a capillary column (HP-5, 0.25 mm × 30 m, 0.25 μm film thickness) under the following GC condition: 1 mL min^{-1} He, on column injection mode, temperature program: 175 ºC for 2 min, thermal gradient 40 ºC min^{-1} to 280 ºC, and then maintained at 280 ºC. *The sample was analyzed by GC-SIM as a bismethaneboronate. **The sample was analyzed by a full-scan GC-MS as a bismethaneboronate.

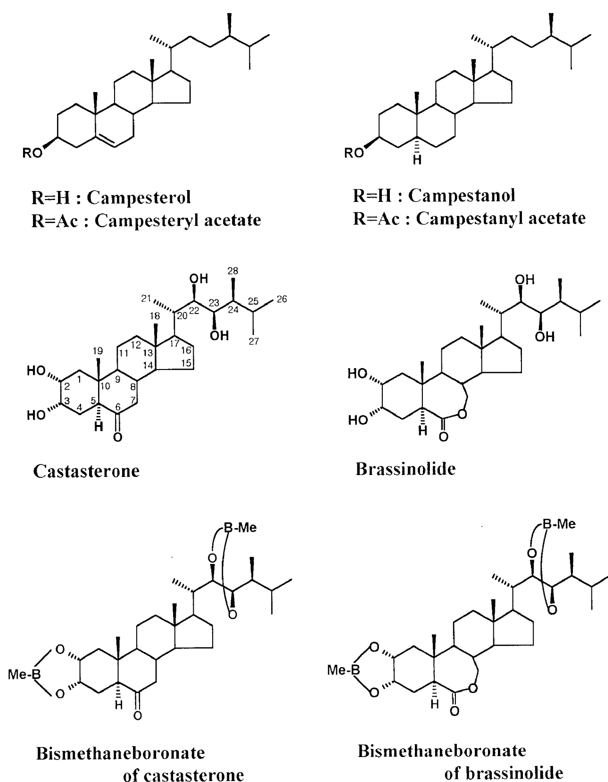

Figure 2. Structures of brassinosteroids and phytosterols identified from the seeds of pumpkin and their derivatives for GC-MS analyses.

expected to contain brassinolide. After methaneboronation, BMB of active compound in the fraction I was analyzed by a full-scan GC-MS. Since no brassinosteroid-like mass spectrum was obtained mainly due to low concentration, active compound in the fraction I was analyzed by GC-SIM (selected ion monitoring). As shown in Table 1, characteristic ions for brassinolide BMB at m/z 528 (M^+), 457, 374, 332, 177, 163 and 155 were co-existed in the identical GC retention time which is the same as that of authentic brassinolide BMB. Therefore, active compound in fraction I was characterized to be brassinolide (Figure 2).

Brassinolide and its direct biosynthetic precursor, castasterone are 24*S*-methylated brassinosteroids which are known to be biosynthesized from campesterol (24*S*-methylcholesterol) *via* the early- and/or late-C6 oxidation pathway.[1~6] Since the co-existence of biosynthetic precursors including campesterol was expected in the seeds of pumpkin, we examined the presence of biosynthetic precursors of castasterone and brassinolide in the same plant material.

The oily residue (50 g) of *n*-hexane soluble fraction was saponified with 80% methanol (500 mL) containing 5% KOH at 70 ºC for 90 min. The unsaponified lipids were extracted with *n*-hexane and charged on silica gel column (Merck, 45 g) chromatography using a mixture (1 : 1) of *n*-hexane and methylene chloride as an elution solvent. Based on the movement on a F254 preparative TLC (developing solvent: ethanol free chloroform), the 4-demethylsterols were separated from non-polar lipids, 4,4-dimethylsterols and 4-methylsterols. The 4-demethylsterols were dissolved in acetic anhydride (1 mL) and pyridine (2 mL) and incubated at room temperature for 18 h. The products were adjusted with HCl solution (pH 3) and extracted with *n*-hexane (200 mL × 3). After removing *n*-hexane, 4-demethylsteryl acetates (0.62 g) were collected and subjected to GC-MS analysis.

24-Methylcholesterol is a common 4-demethylsterol contained in higher plants. In EI (Electron Impact, 70 eV) MS, molecular ion (*m/z* 442) of 24-methylcholesteryl acetate did not appear. Instead, $[M-60]^+$ ion due to fission of acetate at *m/z* 382 was shown as the most prominent ion (base peak). In addition, ions at *m/z* 367, 274, 255 and 213 were detected as characteristic ions which are due to fission of C-C bonds at side chain followed by elimination of acetate.[6] In the GC-MS (EI, 70 eV) analysis of 4-demethylsteryl acetates obtained from the seeds of pumpkin, a peak at 16.11 min on GC showed characteristic ions for 24-methylcholesteryl acetate at *m/z* 382, 367, 274, 255 and 213, indicating the active compound in the peak is 24-methylcholesteryl acetate. To determine configuration of a methyl at C-24, the 4-demethylsteryl acetates were further purified by a reversed phase HPLC using a mixture of methanol and *n*-hexane (97 : 3) as an elution solvent. The obtained 24-methylcholesteryl acetate was then analyzed by 300MHz ^{1}H NMR. The signals for methyls at C-21, -26, -27 and -28 were shown at δ 0.914 (d, *J* =6 .3 Hz), 0.854 (d, *J* = 6.9 Hz), 0.778 (d, *J* = 6.9 Hz) and 0.801 (d, *J* = 6.9 Hz), respectively, which were exactly identical with those of 24*S*-methylcholesteryl acetate, campesteryl acetate. Thus, presence of campesterol in seeds of pumpkin was determined (Figure 2).

Campestanol is a biosynthetic precursor of brassinosteroids which is biosynthesized from campesterol by 5α-reduction at A ring.[14] In the GC-MS analysis of pumpkin 4-demethylsteryl acetates, a peak at 16.23 min on GC gave

Table 2. Identification of phytosterols in *C. moschata* by GC-MS

Compound*	Rt on GC (min)	Prominent ions (m/z, relative intensity)
Endogenous campesterol	16.11	382(100), 367(26), 274(11), 255(13), 213(13)
Endogenous campestanol	16.23	444(22), 384(54), 369(31), 276(34), 257(10), 215(100)
Campesterol	16.11	382(100), 367(26), 274(10), 255(15), 213(17)
Campestanol	16.22	444(25), 384(54), 369(32), 276(25), 257(9), 215(100)

GC-MS (EI, 70 eV) was carried out by Hewlett-Packard 6890-5973 fitted with a capillary column (HP-5, 0.25 mm × 30 m, 0.25 μm film thickness) under the following GC condition: 1 mL min^{-1} He, on column injection mode, temperature program: 175 °C for 2 min, thermal gradient 40 °C min^{-1} to 260 °C, and then maintained at 260 °C. *The sample was analyzed by a derivative of acetate.

molecular ion at *m/z* 444 and characteristic ions at *m/z* 384, 369, 276, 257, 215 which were 2 mass unit increased ions compared with those derived from campesteryl acetate. This suggested that active compound in the peak might be campestanyl acetate. The possibility was confirmed by a direct comparison of mass spectrum and GC retention time with those of authentic campestanyl acetate (Table 2). It was verified that the seeds of pumpkin contain campestanol as a biosynthetic precursor of brassinosteroids (Figure 2).

In summary, two brassinosteroids, castasterone and brassinolide, and their biosynthetic precursors, campesterol and campestanol, were identified from seeds of pumpkin. These steroids are members of the early- and late-C6 oxidation pathway, indicating that the early- and/or late-C6 oxidation pathway are/is contained in the seeds for brassinosteroids biosynthesis (Figure 3). To clarify that, identification of other intermediates in the pathways is under investigation now.

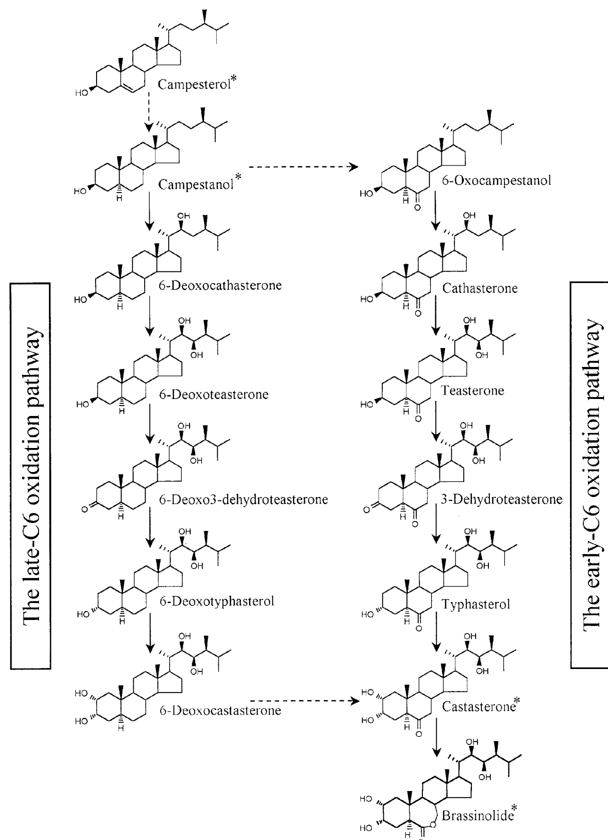

Figure 3. Possible brassinosteroids biosynthetic pathways included in the seeds of pumpkin. Asterisks indicate compounds identified in this study. Broken arrows indicate multiple steps.

The endogenous levels of plant sterols are generally measured in millimoles to micromoles range in plant tissues, and campestanol or 6-oxocampestanol in the early steps of brassinosteroids biosynthetic pathways also exists in plant at similar order of magnitude. On the other hand, brassinosteroids with hydroxyls at side chain occur below nanomolar level in plant, even at pollens or immature seeds in which brassinosteroids are most abundant. This indicates that hydroxylation of the side chain in the biosynthetic pathways must be strictly regulated. In *Catharantus. roseus*, the endogenous levels of cathasterone and downstream intermediates are dramatically reduced to 1/500 that of 6-oxocampesterol, indicating that the hydroxylation at C22 of the side chain would be the limiting step in brassinosteroids biosynthesis.[15]

It has been demonstrated in cells of *Marchantia polymorpha* that a C3-epimerization from teasterone to typhasterol *via* 3-dehydroteasterone, a partial biosynthetic sequence of the early-C6 oxidation pathway, are catalyzed by two cytosolic enzymes, namely teasterone dehydrogenase and 3-dehydroteasterone reductase, respectively.[6] Recently, it has been also demonstrated in *Marchantia* cells that the conversion of typhasterol to brassinolide are mediated by typhasterol 2α-hydroxylase and castasterone oxidase located in the membrane of endoplasmic reticulum (unpublished data). In the late-C6 oxidation pathway, *in vitro* enzymatic conversion of 6-deoxocastasterone to castasterone has been demonstrated in tomato cells by the enzyme produced from *D* gene over-expressed in *E. coli*.[16] All reactions confirmed by enzymatic conversions are relatively late steps of brassinosteroids biosynthesis. To better understand biosynthesis of brassinosteroids, therefore, characteristics of enzymes responsible for the early steps of biosynthetic pathways should be carried out. To this end, preparation of cell-free systems available for the early steps in brassinosteroids biosynthesis, especially in 22*R*- and 23*R*-hydroxylation of brassinosteroids, are underway from seeds of pumpkin.

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