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Brassinosteroids are inherently biosynthesized in the primary roots of maize, *Zea mays* L.

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Abstract

GC-MS analysis revealed that primary roots of maize contain 6-deoxocathasterone, 6-deoxoteasterone and 6-deoxotyphasterol. These brassinosteroids, and the previously identified campesterol, campestanol, 6-deoxocastasterone and castasterone, in the roots are members of a biosynthetic pathway to castasterone, namely the late C-6 oxidation pathway, suggesting that its biosynthetic pathway is operative in the roots. To verify this, a cell-free enzyme extract was prepared from maize roots, and enzymatic conversions from campesterol to castasterone through the aforementioned sterols and brassinosteroids were examined. The presence for the biosynthetic sequences, campesterol → 24-methylcholest-4-en-3β-ol → 24-methylcholest-4-en-3-one → 24-methylcholest-5α-cholestan-3-one → campestanol and 6-deoxoteasterone → 6-deoxo-3-dehydroteasterone → 6-deoxotyphasterol → 6-deoxocastasterone → castasterone were demonstrated. These results indicate that maize roots contain a complete set of enzymes involved in the late C-6 oxidation pathway, thereby demonstrating that endogenous brassinosteroids are biosynthesized in the roots.

Keywords: Biosynthesis; Brassinosteroids; Maize root; The late C-6 oxidation pathway; Gamineae; *Zea mays* L.

1. Introduction

Brassinosteroids (BRs) are steroidal plant hormones that play essential roles in the growth and development of plants. BRs regulate such phenomena as stem elongation, photomorphogenesis, senescence, sex, and vascular organ development (Yokota, 1997; Clouse and Sasse, 1998; Clouse et al., 1996; Li and Chory, 1997). To date, over 40 members of the BR family have been identified within the plant kingdom (Kim, 1991; Fujioka, 1999). Since the first BR, brassinolide (BL), was identified from rape pollen in 1979 (Grove et al., 1979), the presence of BRs has been demonstrated in almost all the aerial parts of plants, pollen, flowers, shoots, vascular cambium, leaves, fruits, and seeds (Kim, 1991; Fujioka, 1999; Fujioka and Sakurai, 1997). It is possible that, in contrast, the identification of BRs in the underground parts of plants, notably the roots, has been delayed, due to comparatively low concentrations of the compounds in these parts. Indeed, in 2000, a study yielded the first identification of a BR, castasterone (CS), **6** (see Fig. 1) in the primary roots of maize (Kim et al., 2000). Recently, the presence of 28-norCS and its biosynthetic precursors, 6-deoxo-28-norcathasterone, 6-deoxo-28-nortyphasterol and 6-deoxo-28-norCS has been demonstrated in tomato roots (Yokota et al., 2001). Therefore, it is now established that plant roots also harbor BRs.

BR-related mutants mainly exhibit their abnormal phenotypes in the aerial organs of plants, but no unique phenotypic changes appear in roots. However, exogenously applied BRs inhibited primary root extension and lateral roots formation (Kawaguchi et al., 1996) and occasionally activate elongation and adventitious rooting, even at concentrations of less than 1 pM (Clouse et al., 1996; Fujioka and Sakurai, 1997; Roddick

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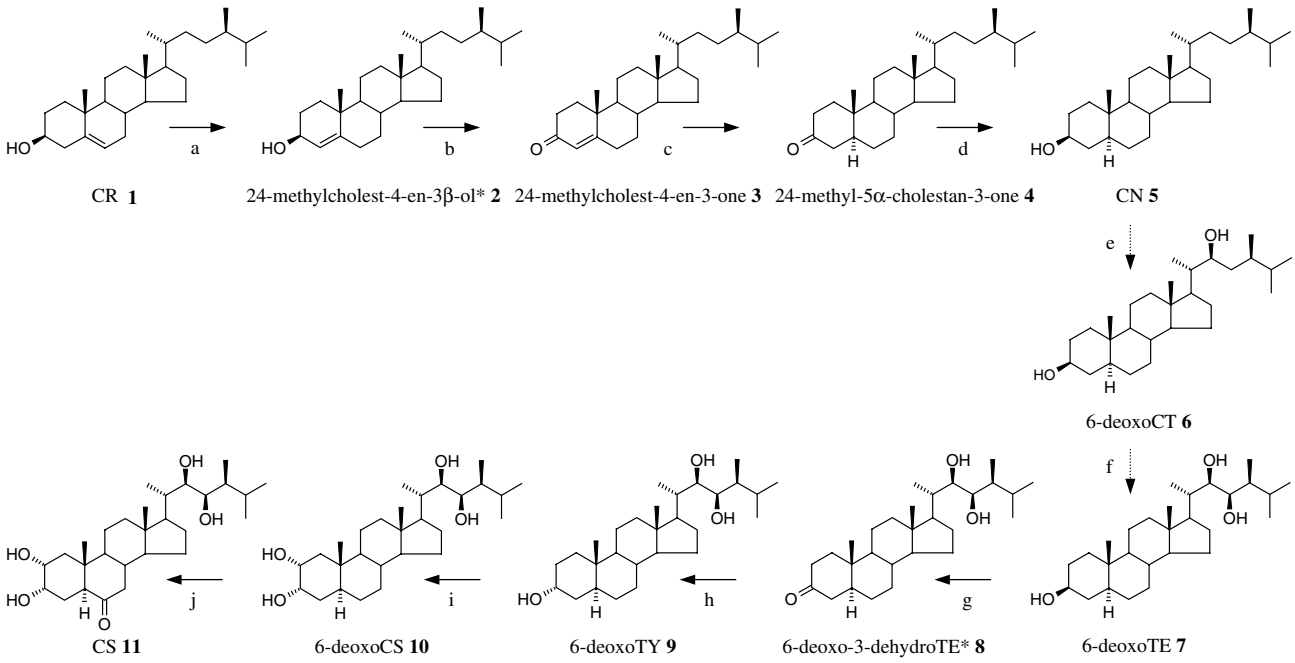

Fig. 1. A biosynthetic pathway for BRs in the primary roots of maize. Asterisks indicate compounds which have not been yet identified. Dashed arrows indicate steps which have not been verified by cell-free enzymatic conversions in this study. a, CR isomerase; b, 24-methylcholest-4-en-3β-ol dehydrogenase; c, 24-methylcholest-4-en-3-one 5α-reductase; d, 24-methyl-5α-cholestan-3-one reductase; e, CN C-22 hydroxylase; f, 6-deoxoCT C-23 hydroxylase; g, 6-deoxoTE dehydrogenase; h, 6-deoxo-3-dehydroTE reductase; i, 6-deoxoTY 2α-hydroxylase; j, 6-deoxoCS oxidase.

and Guan, 1991; Clouse et al., 1993; Sasse, 1994). A BR-insensitive mutant, *bril*, has been isolated from mutagenized *Arabidopsis* plants, in which root elongation was not inhibited by BRs, but by other phytohormones – auxin, gibberellin, cytokinin, abscisic acid, and ethylene, which similar to the situation in the wild-type plant (Clouse et al., 1996). In addition, application of BRs enhanced the gravitropic curvature of maize primary roots, especially in the presence of auxin (Kim et al., 2000). These results indicate that BRs also possess regulatory functions in the growth and development of plant roots.

Although the presence of BRs has been demonstrated in plant roots, the site of their biosynthesis remains obscure. The fact that the same kinds of BRs are identified in both the aerial and underground parts of plants, and that the concentration of BRs in the aerial parts is generally higher than in the underground parts, raises the possibility that BRs in the roots have been transported from the aerial parts. Another possibility is that BRs are inherently biosynthesized in the roots by their own enzyme systems. To shed some light on the bio-origin of BRs in plant roots, we have previously investigated the presence of biosynthetic precursors of CS (**11**) in the primary roots of maize, demonstrating 6-deoxoCS (**10**), campesterol (CR) (**1**), and campestanol (CN) (**5**) in the maize roots (Kim et al., 2004). In addition, a cell-free enzyme extract prepared from maize roots successfully catalyzed the conversion of 6-deoxoCS (**10**) to CS (**11**), suggesting that maize roots are capable of BR-synthesis (Kim et al., 2004). Nevertheless, the possibility of the transport of BRs from the maize shoots to the roots cannot be excluded, as the same sterols and BRs found in the maize roots have been also identified in the shoots (Kang et al., 2003). These questions prompted us to re-investigate the occurrence of additional endogenous BRs in maize roots with a large amount, and to assess the presence of enzymes involved in the biosynthesis of BRs in the roots, which could provide us with concrete evidence of the operation of BR biosynthesis in the roots.

2. Results and discussion

Primary roots of maize were extracted with 80% aq. MeOH, followed by $CHCl_3$. For the purpose of quantitative analysis and facile purification, deuterium-labeled ([26, 28-$^2H_6$])-6-deoxoteasterone (**7**) (6-deoxoTE), -6-deoxocathasterone (**6**) (6-deoxoCT) and -6-deoxotyphasterol (**9**) (6-deoxoTY), which are biosynthetically related to 6-deoxoCS (**10**) and CS (**11**), were added to the $CHCl_3$-soluble extract. The extract was solvent-partitioned, purified by column chromatography, and subjected to reversed-phase HPLC. The HPLC fractions 55–57, 64–66, and 76–80, which corresponded to the retention times of authentic 6-deoxoTE (**7**), 6-deoxoTY (**9**) and 6-deoxoCT (**6**), respectively, under the same HPLC conditions, were analyzed by capillary GC-MS. As a methaneboronate (MB)-trimethylsilylic (TMSi)

ether, an active compound in fraction 55–57 exhibited the same mass spectrum at *m/z* 530 [M^+], 515, 473, 440, 425, 305, 230, 215, and 155, with an equal GC retention time to that of authentic 6-deoxoTE MB-TMSi ether, demonstrating that the compound is indeed 6-deoxoTE (**7**). A MB-TMSi ether of the active compound in fraction 64–66 exhibited an identical mass spectrum at *m/z* 530, 515, 473, 440, 425, 305, 230, 215, and 155 with an equal GC retention time as that of authentic 6-deoxoTY MB-TMSi ether, clearly identifying the active compound as 6-deoxoTY (**9**). The active compound in the fraction 76–80, derivatized to be a di-TMSi ether, did not show the full mass spectrum of 6-deoxoCT di-TMSi ether, but did exhibited the characteristic ions for 6-deoxoCT di-TMSi ether at *m/z* 547 [$M^+ - 15$], 297, 187 (base peak) and 97, with the same GC retention time as that of authentic 6-deoxoCT di-TMSi (Table 1). Therefore, the compound was determined to be 6-deoxoCT (**6**). The endogenous levels of 6-deoxoCT (**6**), 6-deoxoTE (**7**) and 6-deoxoTY (**9**) in the maize roots were determined on the basis of the deuterated standard being 0.1, 1.0 and 9.0 ng g^{-1} fresh weight roots, respectively. Consequently, three BRs- 6-deoxoCT (**6**), 6-deoxoTE (**7**) and 6-deoxoTY (**9**) – were additionally identified from the primary roots of maize.

Both our previous and present studies demonstrated that the primary roots of maize contain CR (**1**), CN (**11**), 6-deoxoCT (**6**), 6-deoxoTE (**7**), 6-deoxoTY (**9**), and 6-deoxoCS (**10**), as well as CS (**11**) (Kim et al., 2004). They are all components of a biosynthetic pathway for the generation of CS (**11**), namely the late C-6 oxidation pathway. This indicates that the pathway is functional in the maize roots. To determine whether or not the late C-6 oxidation pathway is really operating, the presence of activities for enzymes involved in the pathway in the maize roots was assessed using a cell-free crude enzyme solution prepared from the primary roots of maize.

Conversion of CR (**1**) to CN (**5**) involves four biosynthetic steps: CR (**1**) → 24-methylcholest-4-en-3β-ol (**2**) → 24-methylcholest-4-en-3-one (**3**) → 24-methyl-5α-cholestan-3-one (**4**) → CN (**5**) (Noguchi et al., 1999). In order to verify the presence of enzymes catalyzing in these conversions, [$^2H_6$]-CN (**5**) was added as a substrate to the cell-free enzyme solution, incubated at 37 °C for 30 min, and products were then purified by a C_{18} cartridge and reversed-phase HPLC. Under the same HPLC conditions, authentic [$^2H_6$]-24-methylcholest-4-en-3-one (**3**), [$^2H_6$]-24-methyl-5α-cholestan-3-one (**4**) and [$^2H_6$]-CN (**5**) were eluted at 24–25, 29–30 and 24–25 min, respectively. Therefore, the identification of [$^2H_6$]-24-methylcholest-4-en-3-one (**3**) (metabolite I), [$^2H_6$]-24-methyl-5α-cholestan-3-one (**4**) (metabolite II) and [$^2H_6$]-CN (**5**) (metabolite III) in the HPLC fractions 24–25 and 29–30 were carried out by a full scan GC-MS.

As summarized in Table 2, metabolite I manifested prominent ions at *m/z* 404 (M^+, 90), 389 (27), 362 (31), 347 (43), and 229 (100). The mass spectrum and GC retention time (14.45 min) were identical to those observed with authentic [$^2H_6$]-24-methylcholest-4-en-3-one (**3**), demonstrating that metabolite I was, indeed, [$^2H_6$]-24-methylcholest-4-en-3-one (**3**). Metabolite II exhibited the same mass spectrum at *m/z* 406 (M^+, 59), 391 (18), 246 (7), 231 (100), 217 (31), and GC retention time (12.75 min) as did authentic [$^2H_6$]-24-methyl-5α-cholestan-3-one (**4**). Therefore, metabolite II was determined to be [$^2H_6$]-24-methyl-5α-cholestan-3-one (**4**). As a TMSi ether, metabolite III exhibited an equal mass spectrum at *m/z* 480 (M^+, 61), 465 (78), 423 (16), 390 (30), 375 (48) and 215 (100), with the same GC retention time (12.62 min) as dose authentic [$^2H_6$]-CN TMSi, thus proving that metabolite III was [$^2H_6$]-CN (**5**). Consequently, the three metabolites, [$^2H_6$]-24-methylcholest-4-en-3-one (**3**), [$^2H_6$]-24-methyl-5α-cholestan-3-one (**4**) and [$^2H_6$]-CN (**5**) were concluded to be the enzyme products of [$^2H_6$]-CR (**1**). Although [$^2H_6$]-24-methylcholest-4-en-3β-ol (**2**) could not be identified due to the absence of available authentic [$^2H_6$]-24-methylcholest-4-en-3β-ol (**2**), these results indicated that enzymes mediating the conversion of CR (**1**) to CN (**5**), namely, CR isomerase/24-methylcholest-4-en-3β-ol dehydrogenase, 24-methylcholest-4-en-3-one 5α-reductase, and 24-methyl-5α-cholestan-3-one

Table 1
GC-MS data for authentic and endogenous 6-deoxoCT (**6**), 6-deoxoTE (**7**) and 6-deoxoTY (**9**) in primary roots of maize

Compound	*Rt*[c] (min) on GC	Prominent ions (*m/z*, relative intensity %)
Endogenous 6-deoxoCT[a,d] (**6**)	18.19	547 ([$M^+ - 15$], 2), 297 (2), 187 (100), 97 (31)
Authentic 6-deoxoCT[a] (**6**)	18.19	547 ([$M^+ - 15$], 2), 297 (2), 187 (100), 97 (29)
Endogenous 6-deoxoTE[b] (**7**)	19.52	530 (M^+, 44), 515 (41), 473 (22), 440 (23), 425 (33), 305 (31), 230 (26), 215 (100), 155 (38)
Authentic 6-deoxoTE[b] (**7**)	19.52	530 (M^+, 49), 515 (44), 473 (17), 440 (17), 425 (29), 305 (28), 230 (26), 215 (100), 155 (41)
Endogenous 6-deoxoTY[b] (**9**)	16.71	530 (M^+, 28), 515 (5), 473 (4), 440 (59), 425 (57), 305 (12), 230 (30), 215 (100), 155 (22)
Authentic 6-deoxoTY[b] (**9**)	16.71	530 (M^+, 23), 515 (4), 473 (2), 440 (48), 425 (49), 305 (12), 230 (21), 215 (100), 155 (29)

[a] The sample was analyzed as a derivative of trimethylsylilic (TMSi) ether.
[b] The sample was analyzed as a derivative of methanboronate (MB)-TMSi.
[c] *Rt*: Retention time.
[d] GC-Selected ion monitoring (SIM).

Table 2
HPLC and GC-MS data for [$^2H_6$]-CR metabolites in primary roots of maize

Compound	*Rt*[a](min) on HPLC	*Rt*[a] (min) on GC	Prominent ions (*m/z*, relative intensity %)
[$^2H_6$]-CR metabolite I	24–25	14.45	404 (M^+, 90), 389 (27), 362 (31), 347 (43), 229 (100)
[$^2H_6$]-CR metabolite II	29–30	12.75	406 (M^+, 59), 391 (18), 246 (7), 231 (100), 217 (31)
[$^2H_6$]-CR metabolite III[b]	24–25	12.62	480 (M^+, 61), 465 (78), 423 (16), 390 (30), 375 (48), 215 (100)
Authentic [$^2H_6$]-24-methylcholest-4-en-3-one (**3**)	24–25	14.45	404 (M^+, 54), 389 (13), 362 (21), 347 (10), 229 (100)
Authentic [$^2H_6$]-24-methyl-5α-cholestan-3-one (**4**)	29–30	12.75	406 (M^+, 37), 391 (50), 246 (15), 231 (100), 217 (53)
Authentic [$^2H_6$]-CN[b] (**5**)	24–25	12.62	480 (M^+, 26), 465 (35), 423 (13), 390 (34), 375 (35), 215 (100)

[a] *Rt*: Retention time.
[b] The sample was analyzed as a derivative of trimethylsylilic (TMSi) ether.

reductase were all present in the maize roots. The specific enzyme activity for CR isomerase/24-methylcholest-4-en-3β-ol dehydrogenase, 24-methylcholest-4-en-3-one 5α-reductase, and 24-methyl-5α-cholestan-3-one reductase in the roots was approximately 4.3, 13.6 and 808.7 ng mg protein^{-1} min^{-1}, respectively.

Next, the conversion of CN (**5**) to 6-deoxoTE (**7**), via 6-deoxoCT (**6**), was tested with the same enzyme solution. In contrast to the aforementioned enzyme reaction, the conversion of CN (**5**) and 6-deoxoCT (**6**) to their downstream intermediates could not be demonstrated, even in repeated experiments using higher amounts of substrates. In an effort to overcome this problem, only microsomal enzymes were collected from the solution by ultra-centrifugation, and the conversion of CN (**5**) to 6-deoxoTE (**7**) via 6-deoxoCT (**6**) was re-assessed. In spite of these efforts, no reliable amounts of products were detected, even by GC-selected ion monitoring (GC-SIM), indicating that the enzyme activity responsible for mediating C-22 and C-23 hydroxylation in the maize roots is, at best, extremely scarce.

Conversion of 6-deoxoTE (**7**) to 6-deoxoCS (**10**) is intermediated by 6-deoxo-3-dehydroTE (**8**) and 6-deoxoTY (**9**), which is in turn mediated by enzymes, namely 6-deoxoTE dehydrogenase, 6-deoxo-3-dehydroTE reductase, and 6-deoxoTY 2α-hydroxylase. The activity of these enzymes in the maize roots was subsequently examined with cell-free enzyme solution. When [$^2H_6$]-6-deoxoTE (**7**) was used as a substrate, two enzyme products consisting of [$^2H_6$]-6-deoxo-3-dehydroTE (**8**) and -6-deoxoTY (**9**), were detected by GC-SIM analysis (Figs. 2A and B), suggesting the conversion of 6-deoxoTE (**7**) to 6-deoxoTY (**9**) via 6-deoxo-3-dehydroTE (**8**) in the maize root cell-free system. When [$^2H_6$]-6-deoxoTY (**9**) was added to the enzyme mixture, three products were detected by GC-SIM analysis. Of these, two of the products were identified as [$^2H_6$]-6-deoxo-3-dehydroTE (**8**) and -6-deoxoTE (**7**), indicating

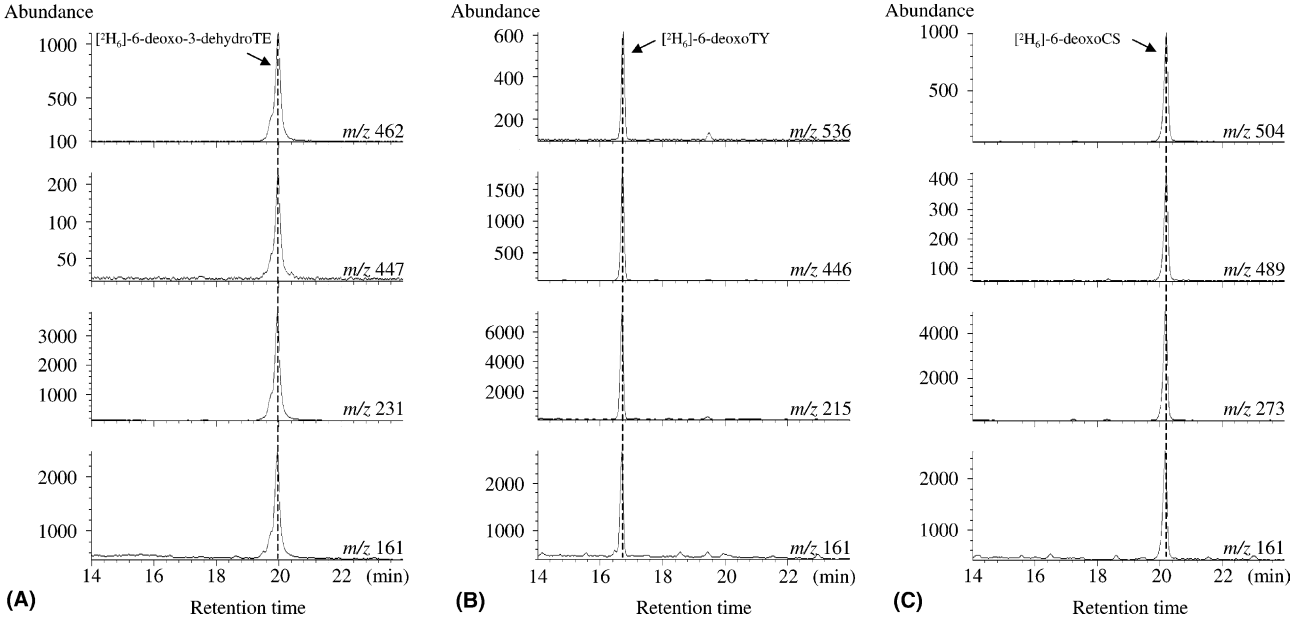

Fig. 2. GC-SIM analyses of enzyme products for 6-deoxoTE dehydrogenase (A), 6- deoxo-3-dehydroTE reductase (B) and 6-deoxoTY 2α-hydroxylase (C) in the primary roots of maize.

that the C-3 epimerization, from 6-deoxoTE (**7**) to 6-deoxoTY (**9**) via 6- deoxo-3-dehydroTE (**8**), is a reversible reaction. The third product was identified as [$^2H_6$]-6-deoxoCS (**10**) (Fig. 2C), demonstrating the C2α-hydroxylation of 6-deoxoTY (**9**) to 6-deoxoCS (**10**). Taken together, this is clear verification of the presence of activity of the three enzymes, 6-deoxoTE dehydrogenase, 6-deoxo-3-dehydroTE reductase and 6-deoxoTY C2α-hydroxylase in the maize roots. The enzyme activity for 6-deoxoTE dehydrogenase, 6-deoxo-3-dehydroTE reductase and 6-deoxoTY C2α-hydroxylase in the roots was approximately 8.4, 58.3 and 3.1 ng mg protein^{-1} min^{-1}, respectively.

Two parallel biosynthetic pathways, namely the early and late C-6 oxidation pathways, are used to produce CS (**11**) and BL from CR (**11**). Both of these pathways have been fully established by feeding experiments using isotope-labeled substrates, and molecular genetic analyses of BR-deficient mutants (Fujioka and Sakurai, 1997; Yokota et al., 1990; Noguchi et al., 2000; Choi et al., 1996). In both pathways, CR is initially oxidized into CN (**5**). In the early C-6 oxidation pathway, CN (**5**) then is converted into to 6-oxoCN, which undergoes successive oxidations to CT, TE, 3-dehydroTE, TY, and CS (**11**). In the late C-6 oxidation pathway, CN (**5**) is oxidized at C-22 to yield 6-deoxoCT (**6**), which then also undergoes successive oxidations to 6-deoxoTE (**7**), 6-deoxo-3-dehydroTE (**8**), 6-deoxoTY (**9**), 6-deoxoCS (**10**), and CS (**11**). In some plants, CS (**11**) is further oxidized into BL, evidence of stronger BR activity in those plants. We have previously demonstrated that the primary roots of maize contain CR (**1**), CN (**5**), 6-deoxoCS (**10**) and CS (**11**) (Kim et al., 2000, 2004). In this study, we additionally identified 6-deoxoCT (**6**), 6-deoxoTE (**7**) and 6-deoxoTY (**9**). All the identified endogenous BRs from the maize roots are constituents of the late C-6 oxidation pathway, implying that CS (**11**), an active BR in the maize roots, is biosynthesized only through the late C-6 oxidation pathway, and not via the early C-6 oxidation pathway. Therefore, the late C-6 oxidation pathway is considered to be the major route in bioactive BR biosynthesis in the maize roots.

IAA is mainly biosynthesized in the shoot apical meristem. The synthesized IAA is then transported to the roots through the phloem, to exert its biological activities in the roots (Choi et al., 1997; Shimada et al., 2001). In contrast, the major plant organ for cytokinin biosynthesis is the roots, from which the cytokinin is transported by xylem streams into the aerial organs (Haberer and Kieber, 2002). In general, a higher level of BRs is detected in the aerial organ of plants, such as pollen, immature seeds and young leaves than in the roots, which bolsters the notion that BRs are mainly biosynthesized in the aerial organs. However, it has recently been demonstrated that the expression of genes encoding CYP85A1/A2 (BRs C-6 oxidase), CYP90A1 (TE or 6-deoxoTE C-23 hydroxylase), CYP90B1 (CT or 6-deoxoCT C-22 hydroxylase), or DET2 (24-methylcholest-4-en-3-one 5α reductase) involved in the early and late C-6 oxidation pathways occurs in both the shoots and roots of *Arabidopsis* seedlings (Li and Chory, 1997; Fujioka and Sakurai, 1997; Noguchi et al., 1999; Shimada et al., 2003; Choe et al., 1998; Szekeres et al., 1996). This suggests that BR biosynthesis is operating not only in the aerial organs, but also the underground portions of plants. This study is to demonstrate, by means of cell-free enzymatic conversions, the presence of enzymatic activity resulting in the conversion of CR (**1**) → 24-methylcholest-4-en-3β-ol (**2**) → 24-methylcholest-4-en-3-one (**3**) → 24-methyl-5α-cholestan-3-one (**4**) → CN (**5**) and 6-deoxoTE (**7**) → 6-deoxo-3-dehydroTE (**8**) → 6-deoxoTY (**9**) → 6-deoxoCS (**10**) in the primary roots of maize. Although the precise enzymes connecting CN (**5**) to 6-deoxoTE (**7**) via 6-deoxoCT (**6**) could not be verified, the fact that 6-deoxoCT (**6**) has been identified as an endogenous BR in maize roots strongly indicates the presence of enzymes catalyzing the conversion from CN (**5**) → 6-deoxoCT (**6**) → 6-deoxoTE (**9**), namely CN C-22 hydroxylase and 6-deoxoCT C-23 hydroxylase, in the roots. Coupled with the previously-verified presence of 6-deoxoCS oxidase (Kim et al., 2004), it is believed that the primary roots of maize may contain a complete set of the enzymes associated with the late C-6 oxidation pathway for the generation of CS (**11**), in which case it is clear concluding that BRs are inherently biosynthesized by their own enzyme system in the maize roots. This study is conclusive evidence for the operation of BR biosynthesis in plant roots.

3. Experimental

3.1. Preparation of primary roots of maize

Maize (*Zea mays* L. cultivar Golden Cross Bantam) seeds were washed with tap water several times, and soaked in distilled water for 24 h. The seeds were then placed on trays (27 × 20 × 2.5 cm) covered by water-saturated paper towels. They were covered with one more layer of water-saturated paper towels in order to keep the seeds moisturized. The trays were positioned vertically at 28 °C in darkness, at 70% relative humidity. After germination in the dark for 2 days, primary roots (2.4 kg), approximately 3 cm in length, were collected from seedlings.

3.2. Purification of BRs in primary roots of maize

The primary roots of maize were extracted with MeOH–H_2O (4:1, 2 L × 3) followed by $CHCl_3$ (1 L × 3). The $CHCl_3$-soluble fraction was dried, and

partitioned between *n*-hexane (1 L) and MeOH–H_2O (4:1, 1 L × 3). The MeOH–H_2O soluble fraction was concentrated into the aqueous phase, and re-partitioned with 0.1 M Pi buffer (pH 7.8) and EtoAc (1 L × 3). The dried EtoAc-soluble fraction (2.5 g) was purified by silica gel CC (Merck, 200 g), and eluted with increasing MeOH content in $CHCl_3$ (0%, 1%, 2%, 3%, 4%, 5%, 7%, 10%, 20%, 50% MeOH in $CHCl_3$, 200 mL each). The biologically active fractions of the 4–5% MeOH in $CHCl_3$ were combined, and loaded onto a Sephadex LH-20 column (22 × 900 mm). They were then eluted with a mixture of MeOH and $CHCl_3$ (1:4). The fractions which eluted between an elution volume/total volume of between 0.65–0.75 were combined, and further purified by reversed-phase HPLC (Senshu Pak Pegasil-B), at a flow rate of 2 mL min^{-1} with a MeCN–H_2O gradient: 0–25 min, 50% MeCN; 25–40 min, 50–100% MeCN; 40–90 min, 100% MeCN. The fractions eluted with the same retention times as [$^2H_6$]-6-deoxoCT (**6**), -6-deoxoTE (**7**) and -6-deoxoTY (**9**) were collected, and then analyzed by GC-MS/SIM after derivatization.

3.3. Enzyme assays

Primary roots of maize (30 g) were ground in a pre-chilled mortar and pestle in 80 mL of 0.1 M Na-Pi buffer (pH 7.4), containing 15 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 40 mM ascorbate, 250 mM sucrose and 10% (v/v) glycerol. The homogenate was filtered and centrifuged at 8000*g* for 15 min. The supernatant was re-centrifuged at 20,000*g* for 30 min. The resulting supernatant was precipitated by the addition of cold Me_2CO, up to a final concentration of 40% (v/v). The supernatant–Me_2CO mixture was kept at −25 °C for 10 min, and then centrifuged at 13,000*g* for 10 min. The resulting precipitate was dissolved in 10 mL of 0.1 M Na-Pi buffer (pH 7.4), containing 1.5 mM 2-mercaptoethanol and 20% (v/v) glycerol, and was used as the cell-free enzyme solution. The protein concentration of the enzyme solution was estimated by a microassay from Bio-Rad using BSA as a standard.

Enzyme assays for the conversion of [$^2H_6$]-CR (**1**) to [$^2H_6$]-deoxoCS (**10**) were initiated by the addition of the substrates (5 μg each), [$^2H_6$]-CR (**1**), -CN (**5**), -6-deoxoCT (**6**), -6-deoxoTE (**7**), and -6-deoxoTY (**9**) to the cell-free enzyme solution (3–4 mg protein mL^{-1}), in the presence of either NADP/NADPH (for the conversion of [$^2H_6$]-CR (**1**) to -CN (**5**)) or NADPH (for the conversion of [$^2H_6$]-CN (**5**) to-6-deoxoCS (**10**) via -6-deoxoCT (**6**), -6-deoxoTE (**7**), -6-deoxo-3-dehydroTE (**8**), and -6-deoxoTY (**9**)). Following incubation at 37 °C for 30 min, enzyme reactions were terminated by the addition of EtOAc (1.2 mL × 3). After confirming that endogenous amounts of sterols and BRs in the enzyme solution were negligible, unlabeled ([$^2H_0$]) expected products were added to the EtOAc-soluble fractions for quantitative analyses. The EtOAc-soluble fractions were concentrated in vacuo, dissolved in MeOH–H_2O (1:1), loaded onto a C_{18} SepPak cartridge column (SepPak plus C_{18}, Waters) and eluted with MeOH–H_2O (1:1) and 100% MeOH (7 mL each, respectively). The fraction eluted with MeOH was dried, dissolved in a small amount of MeOH, and then subjected to reversed-phase HPLC (Senshu Pak C_{18}, 10 × 150 mm) eluted at a flow rate of 2.5 mL min^{-1} with either MeOH for CR (**1**) metabolite or MeCN–H_2O gradients: 0–20 min, 45% MeCN; 20–40 min, 45–100% MeCN; 40–70 min, and MeCN for other metabolites. Fractions were collected every minute. The fractions expected to contain metabolites were then analyzed by capillary GC-MS or GC-SIM following suitable derivatization. The specific enzyme activities were calculated by the ratio of the [$^2H_6$]-product/the [$^2H_0$]-sterols and BRs added as internal standards.

3.4. GC-MS and GC-SIM

GC-MS and GC-SIM analyses were carried out on a Hewlett-Packard 5973 mass spectrometer (Electron impact ionization, 70 electron voltage) coupled to a 6890 gas chromatography fitted with a fused silica capillary column (HP-5, 0.25 mm × 30 m, 0.25 μm film thickness). Oven temperature was maintained at 175 °C for 2 min, elevated to 280 °C at a rate of 40 °C min^{-1}, and then held at 280 °C. Helium was used as the carrier gas, at a flow rate of 1 mL min^{-1} and samples were introduced using an on-column injection mode. Methaneboronation was carried out by heating samples which had been dissolved in pyridine containing methaneboronic acid (2 mg mL^{-1}) at 70 °C for 30 min. *N*-methyl-*N*-TMS-trifluoroacetamide (MSTFA) was used to effect trimethylsilylation.

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References

Choe, S., Dilkes, B.P., Fujioka, S., Takatsuto, S., Sakurai, A., Feldmann, K.A., 1998. The *DWF4* gene of Arabidopsis encodes a cytochrome P450 that mediates multiple 22a-hydroxylation steps in BRs biosynthesis. Plant Cell 10, 231–243.

Choi, Y.H., Fujioka, S., Harada, A., Yokota, T., Takatsuto, S., Sakurai, A., 1996. A brassinolide biosynthetic pathway via 6-deoxocastasterone. Phytochemistry 43, 593–596.

Choi, Y.H., Fujioka, S., Nomura, T., Harada, A., Yokota, T., Takatsuto, S., Sakurai, A., 1997. An alternative brassinolide biosynthetic pathway via late C-6 oxidation. Phytochemistry 44, 609–613.

Clouse, S.D., Sasse, J.M., 1998. Brassinosteroids: essential regulator of plant growth and development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 427–451.

Clouse, S.D., Hall, A.F., Langford, M., McMorris, T.C., Baker, M.E., 1993. Physiological and molecular effects of brassinosteroids *Arabidopsis thaliana*. J. Plant Growth Regul. 12, 61–66.

Clouse, S.D., Langford, M., McMorris, T.C., 1996. A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol. 111, 671–678.

Fujioka, S., 1999. Natural occurrence of brassinosteroids in the plant kingdom. In: Sakurai, A., Yokota, T., Clouse, S.D. (Eds.), Brassinosteroids: Steroidal Plant Hormones. Springer-Verlag, Tokyo, pp. 21–45.

Fujioka, S., Sakurai, A., 1997. Brassinosteroids. Nat. Prod. Rep. 14, 1–10.

Grove, M.D., Spencer, G.F, Rohwededer, W.K., Mandava, N., Worely, J.F., Warthen, J.D., Steffens, G.L., Flippen-Anderson, J.L., Cook, J.C., 1979. Brassinolide, a plant growth promoting steroid isolated from *Brassica napus* pollen. Nature 281, 216–217.

Haberer, G., Kieber, J.J., 2002. Cytokinins, new insights into a classic phytohormone. Plant Physiol. 128, 354–362.

Kang, M.-.W., Kim, Y.-.S., Kim, S.-.K., 2003. Identification and biosynthetic pathway of brassinosteroids in seedling shoots of *Zea mays* L.. Korean J. Plant Biotech. 30, 411–419.

Kawaguchi, M., Imaizumi-Anraku, H., Fukai, S., Syono, K., 1996. Unusual branching in the seedlings of *Lotus japonicus*-gibberellins reveal the nitrogen-sensitive cell division within the pericycle on roots. Plant Cell Physiol. 37, 461–470.

Kim, S.-K., 1991. Natural occurrences of brassinosteroids. In: Cultler, H.G., Yokota, T., Adam, G. (Eds.), Brassinosteroids: Chemistry, Bioactivity and Application, ACS Symposium Series, vol. 474. American Chemical Society, Washington, DC, pp. 26–35.

Kim, S.-K., Chang, S.C., Lee, E.J., Chung, W.-S., Kim, Y.-S., Hwang, S.B., Lee, J.S., 2000. Involvement of brassinosteroids in the gravitropic response of primary root of maize. Plant Physiol. 123, 997–1004.

Kim, T.-W., Chung, W.-S., Kim, Y.-S., Kim, S.-K., 2004. 6-Deoxocastasterone and its biosynthetic precursors from primary roots of maize. Bull. Korean Chem. Soc. 25, 1099–1102.

Li, J.M., Chory, J., 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90, 929–938.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Tax, F.E., Yoshida, S., Feldmann, K.A., 2000. Biosynthetic pathways of brassinolide in *Arabidopsis*. Plant Physiol. 124, 201–209.

Noguchi, T., Fujioka, S., Takatsuto, S., Sakurai, A., Yoshida, S., Li, J., Chory, J., 1999. Arabidopsis *det*2 is defective in the conversion of (24*R*)-24-methylcholest-4-en-3-one to (24*R*)-24-methyl-5a-cholestan-3-one in brassinosteroid biosynthesis. Plant Physiol. 120, 833–840.

Roddick, J.G., Guan, M., 1991. Brassinosteroids and root development. In: Cutler, H.G., Yokota, T., Adam, G. (Eds.), Brassinosteroids: Chemistry, Bioactivity and Application, ACS Symposium Series, vol. 474. American Chemical Society, Washington, DC, pp. 235–245.

Sasse, J.M., 1994. Brassinosteroids and roots. Proc. Plant Growth Regul. Soc. Am. 21, 228–232.

Shimada, Y., Fujioka, S., Miyauchi, N., Kushiro, M., Takatsuto, S., Nomura, T., Yokota, T., Kamiya, Y., Bishop, G.J., Yoshida, S., 2001. Brassinosteroid 6-oxidases from Arabidopsis and tomato catalyze multiple C-6 oxidations in brassinosteroids biosynthesis. Plant Physiol. 26, 770–779.

Shimada, Y., Goda, H., Nakamura, A., Takatsuto, S., Fujioka, S., Yoshida, S., 2003. Organ-specific expression of brassinosteroids-biosynthetic genes and distribution of endogenous brassinosteroids in *Arabidopsis*. Plant Physiol. 131, 287–297.

Szekeres, M., Nemeth, K., Koncz-Kalman, Z., Mathur, J., Kausch-Mann, A., Altmann, T., Redei, G.P., Nagy, F., Schell, J., Knocz, C., 1996. Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. Cell 85, 171–182.

Yokota, T., 1997. The structure, biosynthesis and function of brassinosteroids. Trends Plant Sci. 2, 137–143.

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

Yokota, T., Sato, T., Takeuchi, Y., Nomura, T., Uno, K., Watanabe, T., Takatsuto, S., 2001. Roots and shoots of tomato produce 6-deoxo-28-norcathasterone, 6-deoxo-28-nortyphasterol and 6-deoxo-28-norcastasterone, possible precursors of 28-norcastasterone. Phytochemistry 58, 233–238.