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Biosynthesis and Metabolism of Dolichosterone in *Arabidopsis thaliana*

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The steroidal phytohormones, collectively referred to as the brassinosteroids (BRs), are essential chemical signals that regulate the growth and development of plants.[1-2] Thus far, over 50 naturally-occurring BRs have been identified from the entire plant kingdom.[3] The natural BRs can be classified as C_{27}-, C_{28}-, and C_{29}-BRs based on their carbon skeletons, which basically carry the same carbon numbers as those of the sterols commonly detected in plants.[4] The observed structural similarity between BRs and plant sterols suggests that BRs are biosynthesized from plant sterols, which have similar carbon structures.[5-7] In fact, the results of feeding experiments and molecular genetic studies of BR-related mutants have shown that the most physiologically important C_{28}-BRs, castasterone (Fig. 1, CS) and brassinolide (BL) which have a methyl at C-24, are biosynthesized from 24-methylcholesterol (campesterol) *via* two parallel pathways − namely, the early and late C-6 oxidation pathway − in various plants.[1] We demonstrated recently that the C_{27}-BRs, which harbor no alkyl at C-24, are biosynthesized from a C_{27}-sterol, cholesterol, in young tomato plants in which the presence of C_{28}-BRs biosynthesis has been confirmed.[5] Additionally, we have determined that the end product of C_{27}-BRs biosynthesis, 28-norCS, is converted into CS, thereby indicating that two BRs biosyntheses are connected biosynthetically to maintain the steady-state level of an active C_{28}-BR, CS, in the plant.[5,8] Our continuing interest in the physiological functions of multiple biosyntheses of BRs in plants prompted us to evaluate endogenous BRs in large numbers of *Arabidopsis thaliana,* in which CS and 28-norCS have been characterized. In this paper, the identification, biosynthesis, and metabolism of a 24-methylene BR, dolichosterone (DS), in *A. thaliana* has been reported, which provided us with a clue as to why multiple BR biosyntheses are operant in the plant.

A large quantity (30 Kg) of *A. thaliana* (ecotype *Columbia-0*) was extracted with MeOH and $CHCl_3$. The $CHCl_3$-soluble fraction was dried in vacuo, then partitioned between 80% MeOH and *n*-hexane. The 80% MeOH soluble fraction was partitioned between sodium phosphate buffer (0.1 M, pH 7.8) and ethyl acetate. The obtained neutral ethyl acetate soluble fraction was then concentrated, dissolved in a small amount of MeOH, and loaded onto an SiO_2 column eluted with mixtures of MeOH in $CHCl_3$. The 5 - 7% MeOH in the $CHCl_3$ fraction evidencing biological activity in the rice lamina inclination assay was combined and purified with a Sephadex LH column chromatograph with elution *via* MeOH-$CHCl_3$ = 4:1. The biologically active fractions 24 - 28 were collected, and subjected to reversed-phase HPLC. HPLC fractions 14 - 16, which correspond to authentic DS under the same HPLC conditions were derivatized with methaneboronic acid in pyridine (2 mg/mL), then analyzed *via* capillary GC-selected ion monitoring (SIM). Bismethaneboronate (BMB) of the active principle in HPLC fraction 14 - 16, yielded a molecular ion at *m*/*z* 510 and characteristic ions at *m*/*z* 388, 355, 327, 153 and 124 at the same GC retention time (30.88 min), which are identical to those of authentic DS BMB (Table 1). Therefore, the compound was identified as DS, which

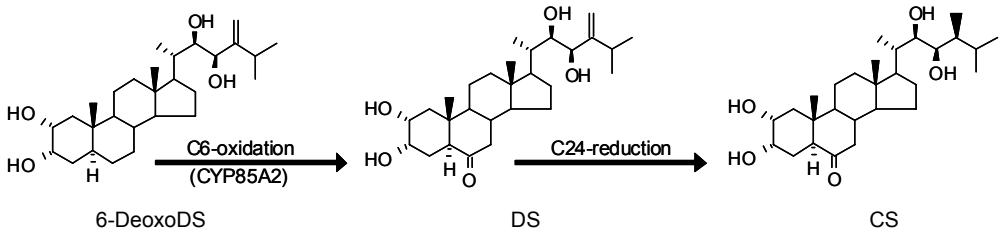

Figure 1. Conversion of 6-deoxoDS to CS intermediated by DS in *A. thaliana*.

Table 1. GC-MS data for authentic and endogenous DS in *A. thaliana*

Compound[a]	Rt[b] on GC	Prominent ions (relative intensity, %)
Athentic DS	30.88	510 (M+, 27), 495 (14), 411 (15), 387 (16), 355/356 (18), 327 (98), 287 (9), 153 (72), 124 (100)
Endogenous DS	30.88	510 (M+, 31), 495 (18), 411 (20), 387 (16), 355/356 (18), 327 (91), 287 (9), 153 (88), 124 (100)

[a]Compound was analyzed as a bismethanemoronate (BMB). [b]Rt: retention time.

[a]These authors contributed equally to this study.

is the first evidence suggestive of the presence of DS in *A. thaliana*.

DS is a 6-ketonic BR that harbors vicinal diols at C-2 and C-3 and C-22 and C-23. In C_{27}- and C_{28}-BRs biosynthesis, the corresponding 6-ketonic BR, 28-norCS, and CS are known to be synthesized *via* the oxidation at C-6 of 6-deoxo-28-norCS and 6-deoxoCS, respectively.[10] Therefore, the conversion of 6-deoxoDS to DS was evaluated with a crude enzyme solution prepared from *A. thaliana*. Following the enzyme assay, the 6-deoxoDS product was purified *via* the procedures described in the Experimental Section, then analyzed by GC-MS/SIM. As a BMB derivative, the product yielded a molecular ion at *m*/*z* 510 and characteristic ions at *m*/*z* 388, 355, 327, 153 and 124. The GC retention time and relative intensities for the mass ions are equal to those derived from synthetic DS BMB. Therefore, the product was identified as DS, thus corroborating that DS can be biosynthesized from 6-deoxoDS in *A. thaliana*.

In *Arabidopsis*, two orthologues of cytochrome P450 − designated as CYP85A1 and CYP85A2 − have been recognized as BR 6-oxidases. In order to determine whether CYP85A1 or/and CYP85A2 catalyze the C-6 oxidation of 6-deoxoDS, CYP85A1, and/or the CYP85A2-deficient mutants, *cyp85a1*, *cyp85a2*, and *cyp85a1*/*cyp85a2* were selected in *A. thaliana*.[1,3] As demonstrated in Fig. 2, the gene expression of CYP85A1 and CYP85A2 is completely diminished in *cyp85a1* and *cyp85a2*, respectively, and the expression of both genes disappears in the double mutant *cyp85a1/cyp85a2*, thereby indicating that the selected mutants are functionally correct. When 6-deoxoDS was added to the crude enzyme solution prepared from *cyp85a1*, a slightly reduced conversion from 6-deoxoDS to DS was detected as compared to that observed in the wild-type variant (Fig. 2b). When 6-deoxoDS was added to the enzyme solution prepared from *cyp85a2*, only a trace of conversion was detected. When 6-deoxoDS was added to *cyp85a1/cyp85a2*, no 6-oxidation of 6-deoxoDS was detected. These findings demonstrate that CYP85A2 is principally responsible for the 6-oxidation of 6-deoxoDS to DS in *A. thaliana*.

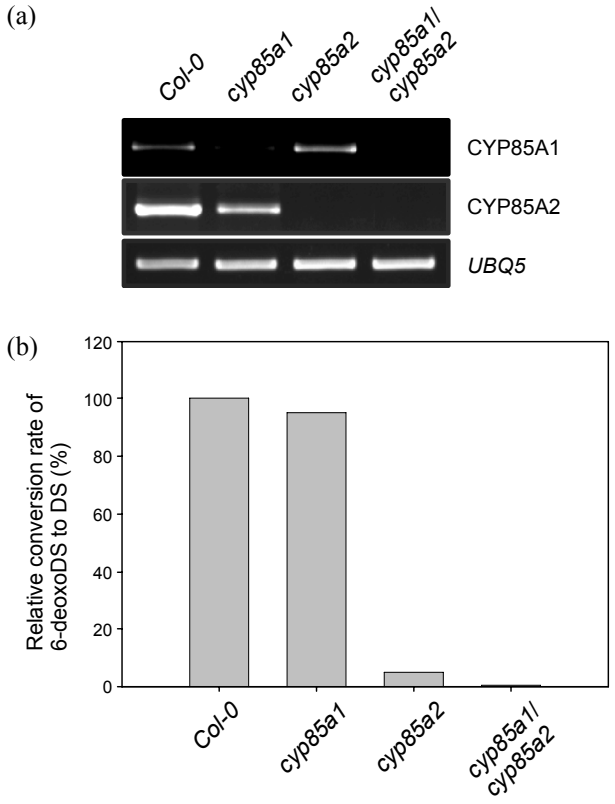

Figure 2. RT-PCR analysis of *cyp85a1*, *cyp85a2* and *cyp85a1/cyp85a2* mutants. *UBQ5* was used as a control (a). Conversion of 6-deoxoDS to DS by Arabidopsis *cyp85a1*, *cyp85a2* and *cyp85a1/cyp85a2* mutants (b).

The metabolism of DS was evaluated using the enzyme solution prepared from wild-type *Arabidopsis* plants. The enzyme assay and product purification were conducted *via* the aforementioned methods, and the product was identified *via* GC-MS analysis. When DS was employed as a substrate in the presence of NADPH, the BMB of a product evidenced a molecular ion at *m*/*z* 512. Prominent ions were detected at *m*/*z* 441, 358, 327, 287, and 155. These molecular and fragment ions are identical to those of CS BMB (Table 2). Furthermore, the GC retention time of the BMB of the product is exactly identical to that of CS BMB. Therefore, CS was identified as the enzyme product of DS, thereby implying that DS is reduced to CS by the incorporation of two protons at C-24.

This study is, to the best of our knowledge, the first to demonstrate the presence of DS in *A. thaliana*. Coupled with the presence of 24-methylene-cholesterol, this finding implies that a biosynthetic pathway from 24-methylene-cholesterol to DS *via* sterols and BRs carrying 24-methylene − namely 24-methylene BRs biosynthesis − is operant in *A. thaliana*. Previously, the presence of C_{27}-BRs biosynthesis resulting in 28-norCS and C_{28}-BRs biosynthesis and the subsequent synthesis of CS and BL has been previously demonstrated in *A. thaliana*.[3] Therefore, multiple biosynthetic pathways (at least three) are functional in *Arabidopsis* plants. In the tomato, 28-norCS is converted into CS with the aid of *S*-adenosylmethionine and NADPH.[10] Recently, we determined that the same C-24 methylation also occurs in *A. thaliana* (published elsewhere). These findings demonstrate that C_{27}-BRs biosynthesis is a biosynthetic pathway that generates biologically active BR and CS in plants. In that study, we demonstrated that DS can be converted to CS *via* an *in vitro* enzyme conversion experiment, thereby suggesting that 24-methylene BRs biosynthesis is also an alternative biosyn-

Table 2. GC-MS data for products assayed by a crude enzyme solution prepared from *A. thaliana*

Substrate[a]	Product[a]	Rt[b] on GC	Prominent ions (relative intensity, %)
6-DeoxoDS	DS	30.88	510 (M+, 27), 495 (14), 411 (15), 387 (16), 355/356 (18), 327 (98), 287 (9), 153 (72), 124 (100)
DS	CS	32.10	512 (M+, 80), 441 (20), 399 (18), 358 (33), 327 (12), 287 (32), 155 (100)

[a]The product was analyzed as a bismethanemoronate (BMB). [b]Rt: retention time

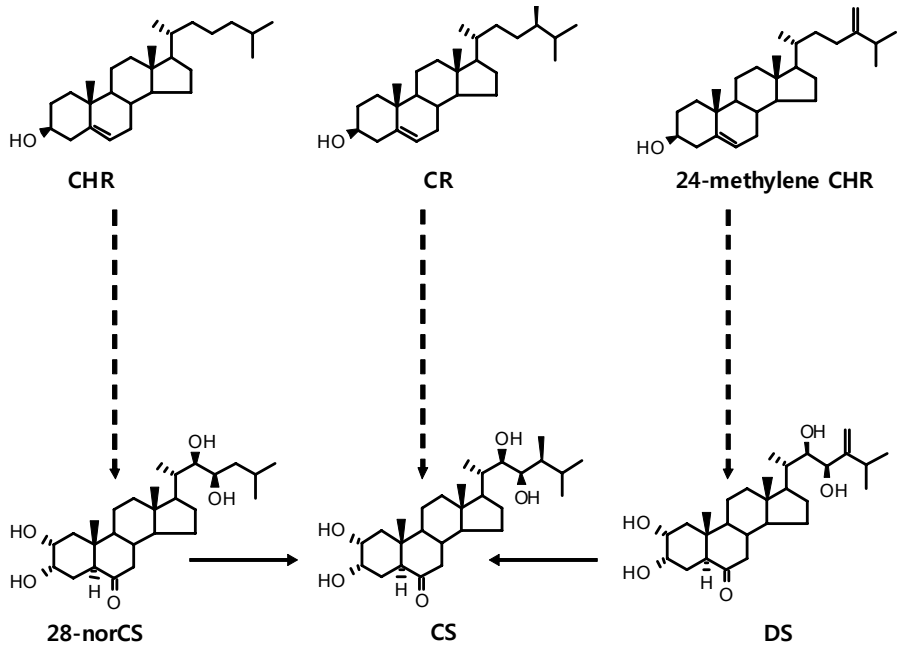

Figure 3. Biosynthetic pathways of C_{27}- and C_{28}-BRs and their connections established in *A. thaliana*.

thetic pathway for the production of CS in *A. thaliana*. Collectively, our results demonstrate that multiple BRs biosynthetic pathways are funneled into CS to maintain high endogenous levels of CS in *A. thaliana* (Fig. 3).

The conversion of 6-deoxoDS to DS was previously demonstrated in *Phaseolus vulgaris* from which both 6-deoxoDS and DS have also been identified.[11] Although the occurrence of 6-deoxoDS has not thus far been noted, *in vitro* conversion using a crude enzyme solution prepared from *A. thaliana* suggests that the 6-oxidation of 6-deoxoDS to DS also occurs in the plant. According to the fact that 6-deoxoCS and 6-deoxo-28-norCS are direct biosynthetic precursors in the late C-6 oxidation pathway for CS and 28-norCS, respectively and the late C-6 oxidation pathway for DS production is likely to be functioning in *Arabidopsis*. Recently, heterologously expressed CYP85A2 in the WAT21 strain of yeast was shown to catalyze the conversion of 6-deoxoDS to DS, but CYP85A1 does not appear to do so.[12] In this study, we also demonstrated that *cyp85a1* catalyzed this conversion, but *cyp85a2 and cyp85a1*/*cyp85a2* did not, thereby indicating that CYP85A1 and CYP85A2 have different affinities against 6-deoxoDS, and that CYP85A2 is mainly responsible for the 6-oxidation of 6-deoxoDS to DS in *A. thaliana*. Coupled with the previous findings that CYP85A2 more strongly catalyzed the conversion of 6-deoxoCS to CS and 6-deoxo-28-norCS to CS, CYP85A2 is believed to represent a more powerful BR 6-oxidase, which is universally involved in multiple BR biosyntheses in plants.

Arabidopsis DWARF1 has been shown to catalyze the reduction of 24-methylenecholesterol to 24-methylcholesterol.[13] Hong *et al.* recently reported that the rice BR-deficient *dwarf2* mutant, which is defective in the rice homologue of *Arabidopsis* DWARF1, accumulates DS, which has not been detected in wild-type rice.[14] These findings indicate that *Arabidopsis* DWARF1 may prove to be responsible for the C-24 reduction of DS to CS in *A. thaliana*. To confirm this possibility, biochemical and molecular genetic analyses of the *Arabidopsis dwarf1* mutant are currently underway.

Experimental Section

Plant materials. Intact plants of *Arabidopsis thaliana* (ecotype *Columbia-0*) grown in a greenhouse were employed in an effort to identify endogenous BRs. The T-DNA insertional mutants, *cyp85a1* and *cyp85a2*, were obtained from ABRC (Columbus, OH). Single genes homozygous for *cyp85a1* and *cyp85a2* were selected according to the previously described methods. A double mutant, *cyp85a1*/*cyp85a2*, was obtained from Dr. Z. Wang at the Carnegie Institution in Stanford University.

Identification of DS in *A. thaliana*. A large amount (30 Kg) of *A. thaliana* was extracted with MeOH (18 L × 5) and subsequently extracted again with $CHCl_3$ (10 L × 5). The $CHCl_3$ soluble fraction (183 g) was concentrated in vacuo, then partitioned between 80% MeOH (1 L × 3) and *n*-hexane (1 L × 3). The 80% MeOH soluble fraction (66 g) was reduced to an aqueous phase, and partitioned between Na-phosphate buffer (1 L × 3) and ethyl acetate (1 L × 3). The obtained neutral ethyl acetate soluble fraction (35 g) was chromatographed on SiO_2 (100 g) eluted with mixtures of $CHCl_3$-MeOH by increasing the MeOH concentration (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100%). The 5 - 8% MeOH in the $CHCl_3$ fraction (5 g) which evidenced biological activity in the rice lamina inclination assay were combined, then loaded on a Sephadex LH column (250 mL) with eluted MeOH-$CHCl_3$ (4:1) as a mobile phase. Fractions were collected every 10 mL. The 24 - 28 fractions were collected (0.5 g), and purified *via* C_{18} column (100 mL) chromatography eluted with aqueous MeOH (50, 60, 70, 80, 90, 100%, 100 mL each). The 80% MeOH fraction (0.05 g) was purified *via* reversed-phase HPLC (Senshu-Pak C_{18}, 10 × 150

mm) eluted with aqueous MeOH as a mobile phase (0 - 20 min: 45%, 20 - 40 min: gradient to 100%, 40 - 60 min: 100% methanol) at a flow rate of 2.5 mL min^{-1}. Fractions were collected every minute. The HPLC fractions corresponding to DS (14 - 16 min) were collected.

Enzyme preparation and assay. Wild-type and BR-deficient mutants (10 g each) were ground in a mortar and pestle with cold 0.1 M sodium phosphate (pH 7.4) buffer containing 15 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 40 mM ascorbate, 250 mM sucrose, and 10% (v/v) glycerol. The homogenates were centrifuged for 15 min at 8,000 g to remove any cell debris. The supernatants were re-centrifuged for 30 min at 20,000 g. The resultant supernatant was then precipitated *via* the addition of cold acetone to a final concentration of 40% (v/v). The supernatant-acetone mixture was maintained at −20 °C for 10 min, then centrifuged for 10 min at 13,000 g. The resultant precipitate was subsequently dissolved in assay buffer containing 0.1 M sodium phosphate (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 20% (v/v) glycerol, then employed as the cell-free enzyme solution.

Reactions were initiated *via* the addition of substrates (6-deoxoDS and DS), followed by 30 min of incubation at 37 °C. The metabolites of the enzyme reactions were extracted using ethyl acetate (1.2 mL) and concentrated in vacuo. The ethyl acetate-soluble fraction was loaded onto a C_{18} Sep-Pak cartridge column, then sequentially washed with 50% and 60% MeOH (5 mL each). The fraction eluted with 100% MeOH was concentrated in vacuo, dissolved in 50 uL of MeOH, and subjected to reverse-phase HPLC, as mentioned above. Finally, after methaneboronation, the enzyme products were analyzed *via* capillary GC-MS/SIM.

GC-MS/SIM analysis. GC-MS/SIM analysis was conducted using a capillary GC-MS: a Hewlett-Packard 5973 mass spectrometer (Electron impact ionization, 70 electron voltage) coupled to a 6890 gas chromatograph fitted with a fused silica capillary column (HP-5, 0.25 mm × 30 m, 0.25 μm film thickness). The oven temperature was maintained for 2 min at 175 °C, elevated to 280 °C at a rate of 40 °C min^{-1}, and then maintained at 280 °C. Helium was employed as the carrier gas at a flow rate of 1 mL min^{-1} and the samples were introduced in on-column injection mode.

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