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Metabolism of 28-Homodolichosterone in *Phaseolus vulgaris*

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The brassinosteroids (BRs) are hormonal chemical signals that are required for normal growth and differentiation in plants.[1,2] Thus far, more than 50 BRs have been identified from various plant materials, distributed throughout the entire plant kingdom.[2-3] Among the plants tested, the immature seed of *Phaseolus vulgaris* is one of the richest plant materials; 22 free BRs and 2 BR conjugates have already been characterized from this species.[2,4] Recently, we demonstrated that multiple biosynthetic pathways function in the synthesis of endogenous BRs in plants, and further, that these pathways are biosynthetically connected, enabling them to generate bioactive BR.[5] This implies that multiple biosynthetic pathways are also functional and interconnected in *P. vulgaris*. To confirm this possibility, the *in vitro* conversion of a C_{29}-BR, 28-homodolichosterone, using a crude enzyme solution prepared from young seedlings of *P. vulgaris* was conducted herein.

It has been demonstrated previously that *P. vulgaris* harbors 6-deoxo-28-homodolichosterone (Fig. 1, 6-deoxo-HDS), a direct biosynthetic precursor of 28-homodolichosterone (HDS).[2,4] In an effort to confirm the biosynthesis of HDS from 6-deoxo-HDS, the *in vitro* conversion of 6-deoxo-HDS to HDS was assessed. To that end, *P. vulgaris* seedlings (5 g) were homogenized in a mortar and pestle with cold 0.1 M sodium phosphate (pH 7.4). The homogenates were then 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 for 10 min at −20 °C, and then centrifuged for an additional 10 min at 13,000 × g. The resultant precipitate was then dissolved in assay buffer (0.1 M sodium phosphate, pH 7.4).

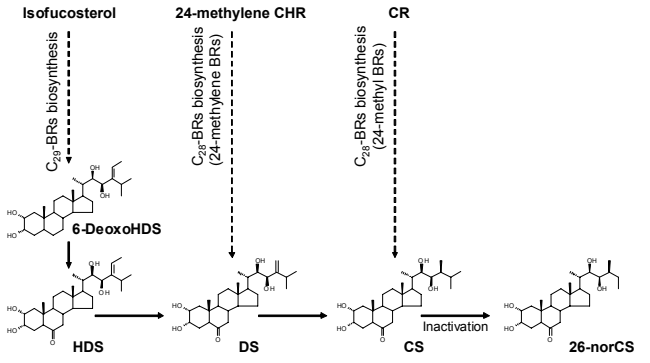

Figure 1. A proposed biosynthetic connection of C_{29}- and C_{28}-BRs biosynthesis in *P. vulgaris*. The dotted arrows indicate multiple steps. The solid arrows indicate a single biosynthetic step which is verified in this study.

The reactions were initiated *via* the addition of 6-deoxo-HDS, and incubated for 30 min at 37 °C. The metabolites of the enzyme reactions were extracted with ethyl acetate (1.2 mL). The ethyl acetate-soluble fraction was then loaded onto a C_{18} Sep-Pak cartridge column, and sequentially washed in aqueous 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 reversed-phase HPLC (Senshu-Pak C_{18}, 10 × 150 mm) eluted with aqueous methanol as a mobile phase (0 - 20 min: 45%, 20 - 40 min: gradient to 100%, 40 - 60 min: 100% MeCN) at a flow rate of 2.5 mL/min. Fractions were collected every min, and the HPLC fractions corresponding to HDS (18 - 20 min) were collected. Finally, the enzyme products were derivatized with methaneboronate in pyridine (1 mg/1 mL) and analyzed *via* GC-MS, using a Hewlett-Packard 5973 mass spectrometer 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).

As summarized in Table 1, the bismethaneboronate (BMB) of the active principal of HPLC fraction 18 - 20 yielded a molecular ion at *m*/*z* 524, and the prominent ions 481, 411, 387, 370, 357, 327, 287, 167, 138 and 123. The mass spectrum and GC retention time at 37.33 min were identical to those of authentic HDS BMB, indicating that the active principal in the HPLC fractions was HDS. This confirms that 28-homodolichosterone can be generated from 6-deoxo-HDS *via* C-6 oxidation in *Phaseolus* plants.

Next, the metabolism of HDS (10 μg) was assessed *via in vitro* enzymatic conversion using the same enzyme solution prepared from the *P. vulgaris* seedlings. After 30 min of assay at 37 °C, the enzyme products were purified by the previously described methods. In the rice lamina inclination assay, biological activities were assessed in fractions 14-17, 19, 20, and 22-24. The active compound in the fractions was derivatized to a BMB, and then analyzed *via* GC-MS. In HPLC fractions 14 and 15, the BMB of the compound evidenced the same mass spectrum and GC retention time as those of synthetic dolichosterone (DS) BMB (Table 1), thereby demonstrating that the compound was DS. In HPLC fractions 16 and 17, as a BMB, the obtained mass spectrum of the active compound was identical to authentic 26-norcastasterone (26-norCS) BMB at an identical GC retention time, thus identifying the compound as 26-norCS. HPLC fractions 19 and 20 corresponded to HDS, which was used as a substrate. As a BMB, the compound in HPLC fractions 22-24 yielded an equal mass spectrum at the same GC retention time to that of castasterone (CS) BMB. Therefore, the compound in the fraction was characterized as CS. As a con-

Table 1. GC-MS analysis for identification of products assayed by a crude enzyme solution prepared from *P. vulgaris*.

Compound[a]		Rt[b] on GC	Prominent ions (relative intensity, %)
Substrate	Product		
6-DeoxoHDS	HDS	35.03	524 (M+, 11), 481 (100), 411 (11), 387 (15), 370 (16), 357 (14), 327 (33), 287 (5), 167 (44), 138 (52),123 (15)
HDS	DS	32.10	510 (M+, 26), 495 (15), 411 (15), 387 (17), 355/356 (20), 327 (97), 287 (10), 153 (72),124 (100)
	CS	31.10	512 (M+, 74), 441 (21), 399 (17), 358 (31), 327 (9), 287 (33), 155 (100)
	26-norCS	27.05	498 (M+, 100), 483 (7), 399 (9), 358 (26), 328 (14), 287 (46), 141 (93)
DS	CS	31.10	512 (M+, 75), 441 (19), 399 (16), 358 (30), 327 (12), 287 (33), 155 (100)
	26-norCS	27.05	498 (M+, 100), 483 (5), 399 (12), 358 (26), 328 (13), 287 (46), 141 (91)
CS	26-norCS	27.05	498 (M+, 100), 483 (8), 399 (9), 358 (27), 328 (10), 287 (42), 141 (91)
Authentic HDS		35.03	524 (M+, 10), 481 (100), 411 (10), 387 (17), 370 (15), 357 (16), 327 (36), 287 (5), 167 (47), 138 (50), 123 (13)
Authentic DS		32.10	510 (M+, 27), 495 (14), 411 (15), 387 (16), 355/356 (18), 327 (98), 287 (9), 153 (72),124 (100)
Authentic CS		31.10	512 (M+, 78), 441 (19), 399 (17), 358 (30), 327 (11), 287 (34), 155 (100)
Authentic 26-norCS		27.05	498 (M+, 100), 483 (8), 399 (10), 358 (27), 328 (11), 287 (42), 141 (93)

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

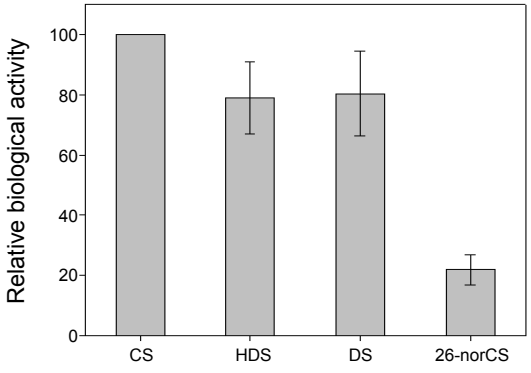

Figure 2. Relative biological activity for CS, DS, HDS and 26-norCS in the rice lamina inclination assay. DS, HDS and 26-norCS shows approximately 20, 20 and 80% less activity compared to that of CS, respectively.

sequence, DS, 26-norCS, and CS were identified as the products of HDS in *P. vulgaris*.

To establish the metabolic sequence of HDS, the metabolism of DS, 26-norCS, and CS were subsequently evaluated *via* the same enzyme assay. When DS was added to the enzyme solution as a substrate, CS and 26-norCS were detected as products by GC-MS analysis (Table 1). When CS was used, 26-norCS was identified as a product. When 26-norCS was added, no BR-like compounds were detected. The conversion rate of HDS to DS, DS to CS and CS to 26-norCS measured by GC-SIM calibration analysis was 3, 4 and 10% in the enzyme assay, respectively. Collectively, the metabolic sequence, HDS → DS → CS → 26-norCS, which is not reversibly converted, was confirmed in *P. vulgaris*.

We demonstrated *via in vitro* enzymatic conversion that CS can be generated from DS in *P. vulgaris*.[6] This implies that two C_{28}-BRs biosyntheses − namely 24-methylene and 24-methyl BRs biosynthesis − are biosynthetically connected, and can produce the physiologically important BR, CS, in plants. In this study, we also demonstrated that HDS can be converted to CS *via* DS in *Phaseolus*. Therefore, a C_{29}-BRs biosynthesis, namely 24-ethylidene BRs biosynthesis, is also likely to be biosynthetically connected to the production of CS *via* DS in plants. In the tomato, 28-norCS, an end product of a C27-BRs biosynthesis, specifically 24-nor BRs biosynthesis, has been identified as a direct precursor of CS.[7] Collectively, a variety of BRs biosyntheses are biosynthetically connected and maintain endogenous CS levels in plants. For this reason, endogenous CS levels are generally higher than those of other BRs in plants.

Biological activity, as assessed *via* rice lamina inclination tests,[8] demonstrates that DS and HDS are less active than CS (Fig. 2). 26-norCS is also biologically less active than CS. Considering the metabolic sequence observed in the *Phaseolus* plants − HDS → DS → CS → 26-norCS, the demethylation of C-28 from HDS intermediated by DS is a biosynthetic reaction that increases BR activity, and the C-26 demethylation of CS to 26-norCS is a catabolic reaction that reduces the activity of an active BR, CS, in *P. vulgaris*.[9]

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