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Characterization of synthetic ecdysteroid analogues as functional mimics of brassinosteroids in plant growth

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ABSTRACT

Brassinosteroids (BRs) are plant steroidal hormones that play important roles in many stages of plant growth. Several plant species produce ecdysteroids, which are known as insect molting steroid hormones. In this study, we evaluated the biological activities of three hydroxysteroidal compounds, 20-hydroxyecdysone (ECD), 7,8-dihydro-8α-20-hydroxyecdysone (DHECD), and 7,8-dihydro-5α,8α-20-hydroxyecdysone (α-DHECD), and compared their activities with that of brassinolide (BL), the most potent BR. In rice, DHECD and α-DHECD enhanced the degree of lamina inclination, as do BRs. In *Arabidopsis thaliana*, DHECD and α-DHECD increased hypocotyl length in the wild-type, and also partially overcame the hypocotyl shortening in the wild-type caused by 0.3 μM brassinazole, a specific BR biosynthesis inhibitor. DHECD and α-DHECD partially reduced dwarfism in the BR-biosynthesis-deficient mutant *det2*. Treatment with DHECD or α-DHECD downregulated the expression of the BR biosynthesis genes *DWF4* and *CPD*, which are generally, suppressed by BR, and upregulated the expression of *TCH4* and *SAUR-AC1*, which are generally promoted by BR. However, their regulated activities were less effective than BL. Moreover, the 10^{-4} M DHECD and α-DHECD induced the accumulation of dephosphorylated BIL1/BZR1 that enhanced BR signaling as a master transcription factor. In contrast, ECD did not affect rice lamina bending, *Arabidopsis* hypocotyl elongation, the expression levels of BR-related genes and BIL1/BZR1 phosphorylation status. Based on these results, we hypothesize that both DHECD and α-DHECD have functional activities similar to those of BR.

1. Introduction

Brassinosteroids (BRs) are a group of naturally occurring steroidal plant hormones that regulate many stages of the plant life cycle [1,2]. BRs have various effects that influence a wide spectrum of cellular responses, including cell division, cell elongation, xylem differentiation, hypocotyl elongation [3], leaf expansion [4], pollen germination [5], and that are processes related to the regulation of gene expression [6]. BRs are also involved in the resistance of plants to biotic and abiotic factors e.g. cold stress, temperature stress, salt stress, and disease [6,7]. BRs and related compounds have been used to enhance production in various crop species including tomato [5], mung bean [8], and rice [9],

Abbreviations: α-DHECD, 7,8-dihydro-5α,8α-20-hydroxyecdysone; BL, brassinolide; BR, brassinosteroid; BRs, brassinosteroids; Brz, brassinazole; d, day; DHECD, 7,8-dihydro-8α-20-hydroxyecdysone; DMSO, dimethyl sulfoxide; ECD, 20-hydroxyecdysone; NOE, nuclear overhauser effect; qRT–PCR, quantitative real-time PCR; WT, Wild-type *Arabidopsis thaliana*

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and consequently, research has been directed towards the discovery of compounds that mimic BRs, and display improved activities and reasonable production costs.

The concentrations of BRs are very low in many plants, and consequently, the yield of naturally occurring BRs from plants is typically poor [10,11]. For example, a trial extraction of BR from 40 kg of rape (*Brassica napus*) pollen resulted in the isolation of only 4 mg of brassinolide (BL) [10], whereas the extraction of 40 kg of insect galls from chestnut (*Castanea crenata*) yielded only 95 μg of castasterone [11]. Ecdysteroids are generally known as compounds that regulate molting and metamorphosis in insects and crustaceans [12,13], but various plant species also produce these compounds (phytoecdysteroids), which are widely thought to inhibit the feeding of phytophagous insects [12]. In a study of *Vitex glabrata*, a common plant in Thailand, 63 g of the phytoecdysteroid 20-hydroxyecdysone (ECD) was obtained from 4 kg of stem bark [14]. ECD was readily converted to 7,8-dihydro analogues, 7,8-dihydro-8α-20-hydroxyecdysone (DHECD) and 7,8-dihydro-5α,8α-20-hydroxyecdysone (α-DHECD), by catalytic hydrogenation and subsequent base-catalyzed epimerization [15].

In addition to their probable role in inhibiting insect feeding, phytoecdysteroids are thought to be involved in the regulation of developmental and physiological processes in plants [16,17]. The hydroxysteroidal compounds ECD, DHECD and α-DHECD have chemical structures similar to those of BRs, so we hypothesized that they function as BR mimics. ECD acts synergistically with indole-3-acetic acid (a naturally occurring auxin) to elongate wheat coleoptiles [17], in a similar way to the synergistic interaction of BRs with auxin, which causes hypocotyl elongation in *Arabidopsis thaliana* (hereafter *Arabidopsis*), a member of the mustard (Brassicaceae) family [18]. Previous studies in rice have demonstrated that DHECD reduces heat stress with efficiency similar to that of 24-epibrassinolide, one of the BRs used commercially in agriculture [19–21]. DHECD promotes pollen viability, pollen germination [19], shoot biomass, leaf expansion [20], and photosynthetic activity under heat stress in rice [20,21]. In this study we compared the biological activities of ECD, DHECD, and α-DHECD with those of BL, using plant physiological and molecular biological methods. The effects of these chemicals were evaluated through their effects on rice inclination, hypocotyl and root elongation in *Arabidopsis,* the expression BR-related genes, and the phosphorylation of BIL1/BZR1 which is a master transcription factor in BR signaling.

2. Material and methods

2.1. Chemical preparation

Brassinolide (BL, Figs. 1A and S1A), and the hydroxysteroids, ECD (Figs. 1B and S1B), DHECD (Figs. 1C and S1C), and α-DHECD (Figs. 1D and S1D) were used as the test chemicals in this study. BL was purchased from Brassino Co., Toyama, Japan. ECD was a natural hydroxysteroid compound obtained from *Vitex glabrata* stem bark [14]. Then, the catalytic hydrogenation was used to reduce the 7,8-unsaturated bond in the B-ring of ECD. The hydrogenating ECD in ethanol and in the presence of sodium nitrite ($NaNO_2$) by using Pd-C as a catalyst gave 7,8-dihydro-8α-20-hydroxyecdysone (DHECD) [15]. Then, 7,8-dihydro-5α,8α-20-hydroxyecdysone (α-DHECD) was obtained in 77% yield by base-catalyzed (2% aqueous Na_2CO_3) epimerization of DHECD. ^{1}H NMR (400 MHz, C_5D_5N): δ 1.39 (s, 2 × 3H, 26-Me and 27-Me), 1.40 (s, 3H, 19-Me), 1.41 (s, 3H, 18-Me), 1.60 (s, 3H, 21-Me), 1.64 (overlapping signal, 1H, H-15a), 1.85 (overlapping signal, 1H, H-24a), 1.89 (overlapping signal, 1H, H-23a), 1.98 (overlapping signal, 1H, H-12a), 1.99 (overlapping signal, 1H, H-15b), 2.18 (m, 1H, H-23b), 2.32 (br dd, J = 11.6, ca 2 Hz, 1H, H-24b), 2.40 (overlapping signal, 1H, H-1b), 2.42 (overlapping signal, 1H, H-12b), 2.45 (overlapping signal, 1H, H-4b), 2.62 (dt, J = 14.4, 4.2 Hz, H-8), 2.69 (dd, J = 14.4, 14.0 Hz, H-7β), 2.87 (dd, J = 11.4, 3.2 Hz, H-5), 2.92 (overlapping signal, 1H, H-17), 2.96 (overlapping signal, 1H, H-9), 3.88–3.95 (overlapping signal, 2 × 1H, H-3 and H-22), 4.34 (br s, $W_{½}$ = 7.5 Hz, 1H, H-2); ^{13}C NMR (100 MHz, C_5D_5N): δ 18.3 (C-18), 21.4 (C-19), 21.6 (C-21), 21.7 (C-11, C-16), 26.3 (C-4), 27.7 (C-23), 30.2 (C-26), 30.5 (C-27), 32.5 (C-15), 33.0 (C-12), 35.8 (C-10), 40.8 (C-7), 42.0 (C-8), 42.8 (C-9), 43.0 (C-24), 46.8 (C-1), 48.0 (C-13), 50.7 (C-17), 52.3 (C-5), 69.9 (C-25), 70.6 (C-2), 72.4 (C-3), 77.3 (C-20), 77.9 (C-22), 84.1 (C-14), 212.8 (C-6); HR-FABMS (negative ion mode): m/z 481.3166 $[M-H]^-$. Stock solutions of each chemical prepared by dissolving appropriate amounts of the compound in dimethyl sulfoxide (DMSO), were stored at −20 °C. The test concentrations were prepared from the stock solution, as required.

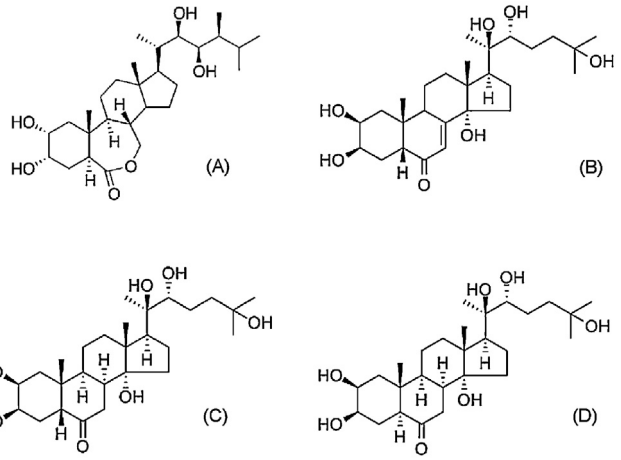

Fig. 1. Chemical structures used in this study. Brassinolide (BL, A), 20-hydroxyecdysone (ECD, B), 7,8-dihydro-8α-20-hydroxyecdysone (DHECD, C), and 7,8-dihydro-5α,8α-20-hydroxyecdysone (α-DHECD, D).

2.2. Rice lamina inclination bioassay

Rice (*Oryza sativa* L.) seeds were grown in the dark over a period of 9 d, and leaf sections consisting of the lamina joint, the lamina 1 cm above the lamina joint, and the leaf sheath 1 cm below the lamina joint were excised for use in the experiment. Solutions (10^{-8}, 10^{-7}, and 10^{-6} M) of BL, ECD, DHECD, and α-DHECD were prepared in 0.1% (v/v) DMSO, and the leaf sections were soaked for 48 h in each BR-related solution. Leaf sections soaked in 0.1% (v/v) DMSO were used as the controls. The inclination angle of the lamina joint was measured with a semicircular protractor, and the concentrations of ECD, DHECD, and α-DHECD causing the greatest inclination angle were selected for use in subsequent experiments.

2.3. Plant materials, growth conditions, and morphological measurements

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type plant. The *Arabidopsis* BR-biosynthesis *det2* mutant was selected as a BR mutant plant showing dwarfism. Seeds were germinated on 1/2 Murashige and Skoog (MS) medium containing 0.8% phytoagar (Duchefa, Haarlem, The Netherlands) and 1.5% sucrose. The medium was supplemented with 0.1% (v/v) DMSO (as the control) or the test chemicals 10^{-8} M BL, 10^{-6} M ECD, 10^{-6} M DHECD, or 10^{-6} M α-DHECD. Plants were grown in the control or treatment medium at 22 °C in the dark for 10 d. The hypocotyl and root lengths were measured with the ImageJ software.

2.4. Quantitative real-time PCR

Wild-type *Arabidopsis* (WT) and *det2* mutant plants were grown in the dark for 7 d in 1/2 MS medium contained 0.8% phytoagar and 1.5% sucrose. The plants were soaked for 3 h in 0.1% (v/v) DMSO (control), 10^{-8} M BL, 10^{-6} M ECD, 10^{-6} M DHECD, or 10^{-6} M α-DHECD solutions prepared in 1/2 MS medium without phytoagar and sucrose. Plant

samples were removed and stored in liquid nitrogen for later RNA extraction. Total RNA was extracted from the samples with the RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). The complementary DNA (cDNA) was synthesized using PrimeScript (Takara, Kyoto, Japan), and was used in quantitative real-time PCR (qRT–PCR). qRT–PCR was performed according to the instructions provided with the PCR Thermal Cycler Dice (Takara, Tokyo, Japan), using the SYBR Premix ExTaq system (Takara, Shiga, Japan).

The following primers were used: *DWF4*-forward 5′-CATAAAGC TCTTCAGTCACGA-3′ and *DWF4*-reverse 5′-CGTCTGTTCTTTGTTTCC TAA-3′; *CPD*-forward 5′-CACTTCAAAGATGCTCGCACTT-3′ and *CPD*-reverse 5′-CAGCTCGTAACCGGGACATAG-3′; *TCH4*-forward 5′-CGAGT CTTGGAACGCTGAT-3′ and *TCH4*-reverse 5′-CTTCTTGTTGAAAGCCA CGG-3′; *SAUR-AC1*-forward 5′-GAGATATGTGGTGCCGGTTT-3′ and *SAUR-AC1*-reverse 5′-GTATTGTTAAGCCGCCCATT-3′; and *ACT2*-forward 5′-CGCCATCCAAGCTGTTCTC-3′ and *ACT2*-reverse 5′-TCACGT CCAGCAAGGTCAAG-3′. *ACT2* was used as the constitutively expressed control gene.

2.5. Western blot analysis

Wild-type *Arabidopsis* (WT) and *BIL1/BZR1-GFP* transgenic plants were grown under light condition for 7 d in 1/2 MS medium with 1 μM Brz. The plants were treated with 0.1% (v/v) DMSO (control), 10^{-8} M BL, 10^{-5} M and 10^{-4} M of ECD, DHECD, or α-DHECD prepared in 1/2 MS medium for 3 h. Then, plants were collected to extract protein by boiling with twice volume per fresh weight of 1 × Laemmli buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% [w/v] SDS, 0.1% [w/v] bromophenol blue, and 10% [w/v] glycerol). The proteins were separated by electrophoresis using SDS-PAGE (10% acrylamide gel). The electrophoretical proteins were transferred to a nitrocellulose blotting membrane (Amersham, Buckinghamshire, UK) and were blocked in TBS (Tris-Buffered Saline) containing 3% skim milk at room temperature. The nitrocellulose membrane was incubated overnight at 4 °C in Western Blot Immuno Booster Solution I (Takara, Tokyo, Japan) with a polyclonal antibody (1:20,000) against GFP (Molecular Probes). After that membrane was washed in TBS containing 1% skim milk at room temperature and was incubated in Western Blot Immuno Booster Solution II (Takara, Tokyo, Japan) with horseradish peroxidase-conjugated secondary antibody (1:50,000; Promega) for 1 h at room temperature. The BIL1/BZR1-GFP polypeptide was detected by the LAS-4000 mini (Fujifilm, Tokyo, Japan). Images were analyzed by using Multi Gauge Ver3.0 software (Fujifilm, Tokyo, Japan) to determine the relative signal intensity. Dephosphorylated BIL1/BZR1 to phosphorylated BIL1/BZR1 ratios (de P-BIL1/P-BIL1) were calculated from their signal intensities.

3. Results

3.1. DHECD and α-DHECD produced rice lamina inclination like BRs

BL is known to strongly induce rice lamina inclination. The lamina joint of rice was treated with various concentration of BL, ECD, DHECD, or α-DHECD, and the angle of inclination of the lamina was measured. BL, DHECD, and α-DHECD induced significant lamina bending, with the angle dependent on the concentration of the chemical (Fig. 2), but the effect of BL was greater. The lamina angle in rice treated with ECD was not significantly different from that of the control (Fig. 2). This result suggests that DHECD and α-DHECD should have BR-like activity in rice lamina inclination. In the further experiments we used chemical concentration that caused the highest degree of lamina inclination. As 10^{-6} M of all compounds showed the most lamina bending activity, we tested those compounds at this concentration (Fig. 2). However, 10^{-6} M BL strongly reduced hypocotyl length of wild-type *Arabidopsis* (Fig. S2A) and BL significantly decreased root length associated with concentrations (Fig. S2B). Because BL clearly showed the inhibition of root elongation, we used this parameter to calculate the half inhibitory concentration (IC_{50}) of each compounds by IC_{50} Tool Kit from http://ic50.tk/index.html. The results showed that IC_{50} of BL, ECD, DHECD and α-DHECD were $\leq 10^{-10}$, $\geq 10^{-4}$, 1.16×10^{-5} and 1.04×10^{-5} M, respectively (Fig. S3). It was confirm that BL at 10^{-6} M is toxic on *Arabidopsis* root. Based on this reason, 10^{-8} M BL was used instead of 10^{-6} M BL in the following experiments.

3.2. DHECD and α-DHECD increase Arabidopsis hypocotyl length in the dark

In wild-type *Arabidopsis*, 10^{-8} M BL treatment significantly increased the hypocotyl length, but reduced the root length, whereas 10^{-6} M DHECD and 10^{-6} M α-DHECD increased both the hypocotyl and root lengths. However, ECD had no effect on hypocotyl or root elongation in *Arabidopsis* (Table 1A, Fig. S4A). We used the specific BR biosynthesis inhibitor brassinazole (Brz) [22,23] to clarify the functions of ECD, DHECD, and α-DHECD as BR mimics. The results showed that 0.3 and 3 μM Brz significantly reduced the hypocotyl length in wild-type *Arabidopsis*, but this effect was reversed by the application of BL. DHECD and α-DHECD also reversed this effect in wild-type *Arabidopsis* treated with 0.3 μM Brz, but not in the plants treated with 3 μM Brz. ECD had no effect on hypocotyl length at either Brz concentration (Table 1B and C, Fig. S4B and C). Although the BL treatment reversed the dwarf phenotype of the wild-type *Arabidopsis* hypocotyls treated with Brz, it significantly reduced the root length. In contrast, DHECD or α-DHECD caused a significant increase root length of wild-type *Arabidopsis* treated with Brz (Table 2; Fig. S4B and C).

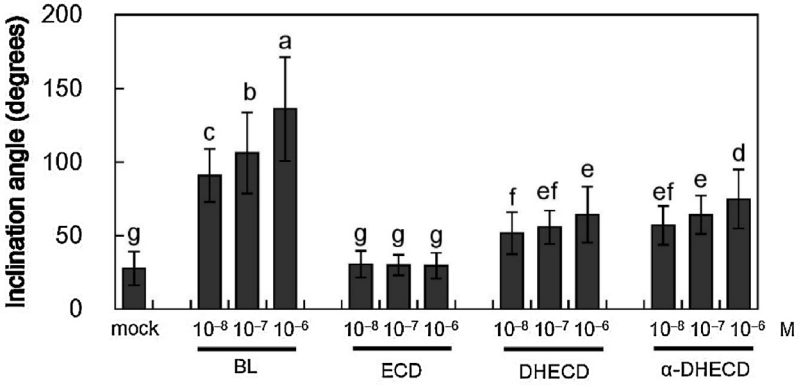

Fig. 2. DHECD and α-DHECD have the weak effect on the increase of rice lamina inclination. Data are the means of 30 replicates. Standard deviations are shown as vertical error bars. Means followed by the same letter are not significantly different at $P \leq 0.05$ (Duncan's multiple range test).

Table 1
Effects of BL, ECD, DHECD, and α-DHECD on hypocotyl and root length in wild-type *Arabidopsis* without Brz (A) and wild-type *Arabidopsis* treated with 0.3 μM Brz (B) or 3 μM Brz (C) grown in the dark for 10 d.

Treatment	Hypocotyl length (mm)[a]	Root length (mm)[a]
(A) Control	17.46 ± 2.38b	13.82 ± 2.20b
BL 10^{-8} M	21.32 ± 1.56a	6.21 ± 1.41c
ECD 10^{-6} M	17.87 ± 2.47b	13.42 ± 2.40b
DHECD 10^{-6} M	20.74 ± 2.23a	15.26 ± 2.12a
α-DHECD 10^{-6} M	21.06 ± 1.51a	15.75 ± 2.39a
(B) Control	17.46 ± 2.38a	13.82 ± 2.20b
Brz 0.3 μM	7.29 ± 1.21d	11.41 ± 2.26c
BL 10^{-8} M + Brz 0.3 μM	17.26 ± 1.90a	7.22 ± 2.22e
ECD 10^{-6} M + Brz 0.3 μM	7.29 ± 0.77d	10.16 ± 1.65d
DHECD 10^{-6} M + Brz 0.3 μM	10.31 ± 1.56c	14.90 ± 2.00a
α-DHECD 10^{-6} M + Brz 0.3 μM	11.61 ± 1.48b	15.51 ± 2.15a
(C) Control	17.46 ± 2.38a	13.82 ± 2.20a
Brz 3 μM	2.06 ± 0.41c	8.90 ± 1.98b
BL 10^{-8} M + Brz 3 μM	8.42 ± 2.50b	5.95 ± 1.69c
ECD 10^{-6} M + Brz 3 μM	2.17 ± 0.35c	9.02 ± 1.42b
DHECD 10^{-6} M + Brz 3 μM	2.25 ± 0.51c	14.52 ± 2.10a
α-DHECD 10^{-6} M + Brz 3 μM	2.34 ± 0.49c	2.08a

[a] Data are the means of 40 seedlings ± SD. Means followed by the same letter are not significantly different at P ≤ 0.05 (Duncan's multiple range test).

Table 2
Effects of BL, ECD, DHECD, and α-DHECD on hypocotyl and root length in the *Arabidopsis det2* mutant grown in the dark for 10 d.

Treatment	Hypocotyl length (mm)[a]	Root length (mm)[a]
Control	2.86 ± 0.40d	6.68 ± 1.84b
BL 10^{-8} M	11.66 ± 2.51a	4.79 ± 1.26c
ECD 10^{-6} M	2.99 ± 0.70d	5.14 ± 2.08c
DHECD 10^{-6} M	4.11 ± 0.52c	7.42 ± 2.05ab
α-DHECD^{-6} M	4.73 ± 0.73b	7.69 ± 1.98a

[a] Data are the means of 40 seedlings ± SD. Means followed with the same letter are not significantly different at P ≤ 0.05 (Duncan's multiple range test).

3.3. Reversal of BR-deficient dwarfism in Arabidopsis det2 mutant by DHECD and α-DHECD

BR-biosynthesis *det2* mutant was used to investigate the BR mimic activity of ECD, DHECD, and α-DHECD. The treatment of *det2* mutant hypocotyls with 10^{-6} M DHECD or 10^{-6} M α-DHECD reversed the *det2* dwarf phenotype by increasing the hypocotyl length, but to a lesser extent than did 10^{-8} M BL. ECD treatment had no effect on *det2* hypocotyl elongation. The effects of DHECD and α-DHECD on *det2* root length were similar to their effects in wild-type *Arabidopsis*: whereas BL application significantly reduced the root length in the *det2* mutant, both DHECD and α-DHECD increased the root length (Table 2; Fig. S4D).

3.4. DHECD and α-DHECD regulate the expression of BR-related genes

To assess the regulatory effects of ECD, DHECD, and α-DHECD, we investigated the relative expression of BR-responsive genes in *Arabidopsis* treated with these compounds. qRT–PCR showed that the expression of BR biosynthesis genes (*DWF4* and *CPD*) in BL-treated wild-type *Arabidopsis* was significantly downregulated by negative feedback [24,25]. Moreover, in BL-treated wild-type *Arabidopsis*, the expression of *TCH4* (a xyloglucan-endotransglycosylase-encoding gene that is usually induced by BR treatment) and *SAUR-AC1* (an early auxin-inducible gene that is regulated independently by BR) was upregulated [24,25]. Conversely, the expression of *DWF4* and *CPD* was upregulated in BR-deficient plants, including Brz-treated wild-type *Arabidopsis* and the BR-biosynthesis *det2* mutant. Similarly, the expression of *TCH4* and *SAUR-AC1* was downregulated in the BR-deficient plants (Fig. 3).

As shown in the physiological analysis based on hypocotyl elongation, the BR-related gene expression patterns in the DHECD- and α-DHECD-treated plants were similar. In these experiments BR-deficient *det2* mutant was more sensitive to BR-treatment stimulating gene expression of *TCH4* and *SAUR-AC1* than wild-type *Arabidopsis*. DHECD and α-DHECD also induced significant expression of the *TCH4* and *SAUR-AC1* genes in the *det2* mutant (Fig. 4C and D). The expression of *DWF4* in *det2* was downregulated by both DHECD and α-DHECD, in a similar way to the downregulation caused by BL treatment (Fig. 3A). Furthermore, the expression of the biosynthesis gene *CPD* in wild-type *Arabidopsis* was also downregulated by DHECD and α-DHECD (Fig. 3B), whereas ECD rarely have no significant effect on BR-related gene expression (Fig. 3). This result parallels those in the experiments based on rice lamina inclination and *Arabidopsis* hypocotyl elongation.

3.5. DHECD and α-DHECD induce the dephosphorylation of BIL1/BZR1 protein

Phosphorylation status of BRZ-INSENSITIVE-LONG HYPOCOTYL 1/BRASSINAZOLE RESISTANT 1 (BIL1/BZR1) is generally used to identify BR-related signaling. BR-treatment induces the dephosphorylation of BIL1/BZR1 that can be detected as a decrease of BIL1/BZR1 molecular weight [26]. To reveal whether ECD, DHECD and α-DHECD mimic BR effects through BR signaling pathway, the phosphorylation states of BIL1/BZR1-GFP protein with these three hydroxysteroidal compounds was analyzed by immune blotting using anti-GFP antibody. Since BR-deficient mutants such as *det2* or BR-deficient plants caused by Brz treatment were more sensitive to BR than Wild-type *Arabidopsis* (WT) or by control treatment respectively, we checked the effect of these compounds on the status of BIL1/BZR1-GFP protein in Brz-treated plants. In this experiment we used three hydroxysteroidal compounds at higher concentrations (10^{-5} M and 10^{-4} M) than in former experiments in expectation of the clear result. The result showed that control treatment of *BIL1-GFP Arabidopsis* gave a low ratio of dephosphorylated BIL1/BZR1 to phosphorylated BIL1/BZR1 (de P-BIL1/P-BIL1). On the other hand, 10^{-8} M BL treatment exhibited a higher ratio of de P-BIL1/P-BIL1 than control treatment (Fig. 4). At 10^{-5} M, the de P-BIL1/P-BIL1 ratio of DHECD- or α-DHECD-treated plants were slightly different form that of the control (Fig. 4), whereas at 10^{-4} M both treatments gave as high de P-BIL1/P-BIL1 ratios as BL treatment (Fig. 4). However, ECD did not show a clear effect on BR signaling even at 10^{-4} M (Fig. 4).

4. Discussion

Since the discovery of BRs and their potential applications, several BR analogues have been synthesized, allowing their large-scale economic production. In this study, we have demonstrated that the hydroxysteroidal compounds, DHECD and α-DHECD mimicked the function of BRs, promoting the rice lamina bending (Fig. 1) and reversing BR-deficient dwarfism in dark-grown *Arabidopsis* seedlings (Table 1B and C). The rice lamina inclination test is a very sensitive bioassay for BRs, so it is useful for assessing whether test compounds have BR-like activity [27,28]. Therefore, both DHECD and α-DHECD were found to have a BR-like effect on lamina inclination, but were less active than BL (Fig. 1).

The hypocotyl length of the wild-type *Arabidopsis* Col-0 treated with BL, DHECD, or α-DHECD was significantly increased (Table 1). Low concentrations of BR (nM to μM concentrations) have been reported to enhance hypocotyl elongation [6,29], resulting from cell enlargement [3]. We found that a high concentration of BL significantly reduced root elongation in wild-type *Arabidopsis*, which differed from the results for both DHECD and α-DHECD (Table 1). Previous reports have suggested that the exogenous application of BR at low concentrations promotes

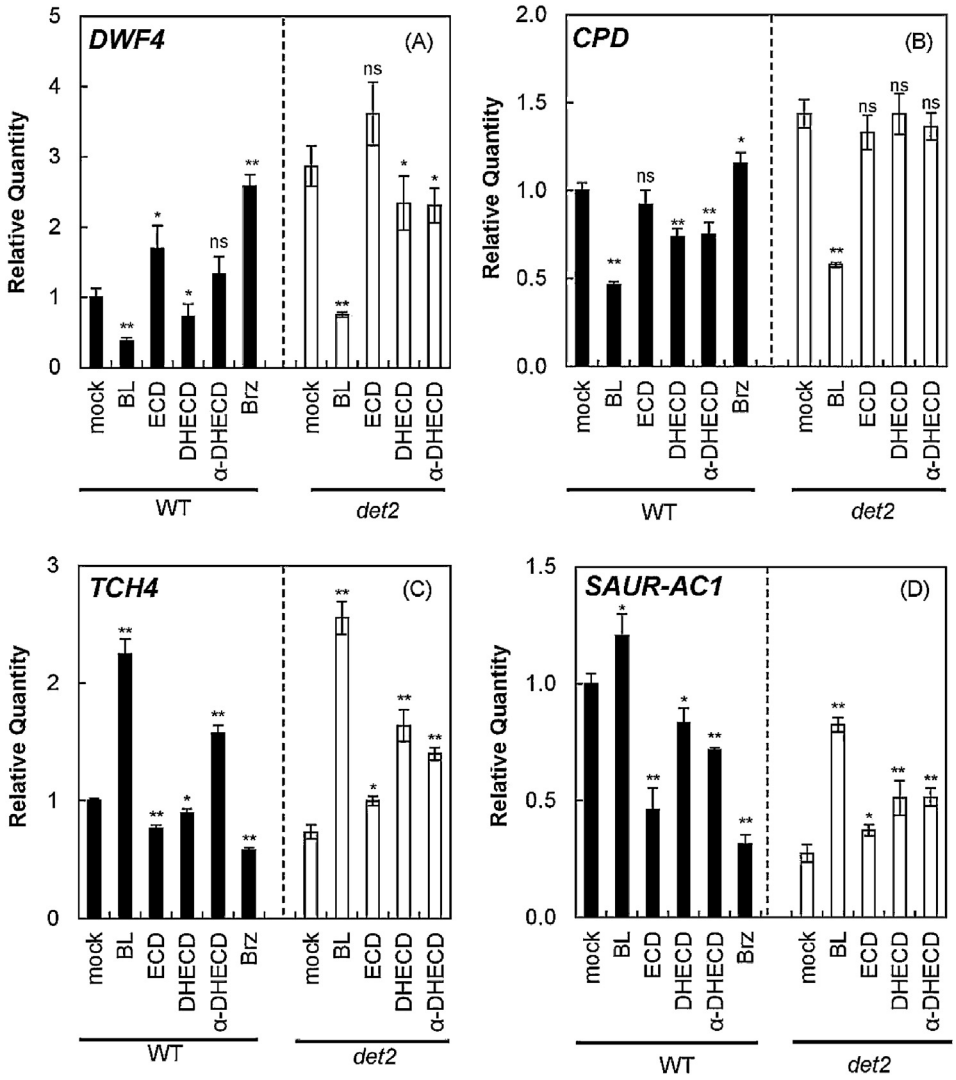

Fig. 3. DHECD and α-DHECD regulate some BR-related genes expression. Real-time PCR analysis of *DWF4* (A), *CPD* (B), *TCH4* (C), and *SAUR-AC1* (D) expression in wild-type *Arabidopsis* (WT) and the BR-biosynthesis *det2* mutant (*det2*) as controls or in plants treated with 10^{-8} M BL, 10^{-6} M ECD, 10^{-6} M DHECD, or 10^{-6} M α-DHECD. Data are the means of four replicates. Standard deviations are shown as vertical error bars. Statistically significant differences relative to the control are $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, and ns: nonsignificant (Student's *t* test).

root elongation, but at high concentrations BRs inhibit root growth [30,31]. Müssig et al. [31] reported that 24-epibrassinolide concentrations that higher than 10^{-9} M exhibited the inhibition of root growth in wild-type *Arabidopsis*. Moreover, IC_{50} of each chemicals showed that BL had higher IC_{50} considering from root inhibition than DHECD and α-DHECD (Fig. S3). It is imply DHECD and α-DHECD concentration had the toxic on root growth lower than BL. This can explain why treatment with DHECD or α-DHECD promoted root elongation, even at high concentrations. As suggested by their effects on hypocotyl elongation, the effectiveness of DHECD and α-DHECD was less than that of BL. Therefore, at high concentrations, these two chemicals may have a similar effect as low concentrations of BR, and promote root elongation. When DHECD, or α-DHECD was applied to hypocotyls in the presence of Brz, the hypocotyl shortening caused by 0.3 μM Brz was reversed (Table 2), but in the presence of 3 μM Brz, the hypocotyl shortening was not reversed (Table 2). Brz is a specific BR

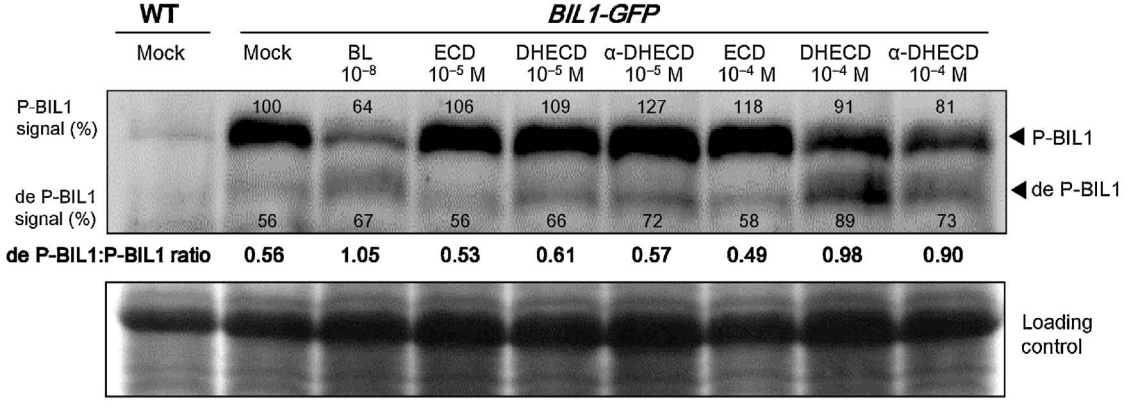

Fig. 4. DHECD and α-DHECD at 10^{-4} M have the same dephosphorylated BIL1/BZR1 to phosphorylated BIL1/BZR1 ratios (de P-BIL1:P-BIL1 ratio) as BL. The signal intensities of P-BIL1 and de P-BIL1 are shown by the top and below arrowheads. Gel was stained with Ponceau to indicate total proteins used as loading control.

biosynthesis inhibitor that inhibits C-22 hydroxylation in the BR biosynthesis pathway [23]. Brz induces dwarfism in many plants, including *Arabidopsis*, but this is reversible by the application of BRs [22,23]. Therefore, our results suggest that DHECD and α-DHECD have the same functions as BRs. We also used the *Arabidopsis det2* mutant to clarify the functions of ECD, DHECD, and α-DHECD. The *det2* mutant is a BR-biosynthesis mutant that produces shorter hypocotyls in the dark than does the wild-type [32,33]. The *det2* phenotype can be reversed by the addition of BRs [34]. The treatment of *det2* mutant plants with DHECD or α-DHECD significantly reversed their dwarfism, although to a lesser extent than BL, whereas treatment with ECD had no effect on their dwarfism (Table 2). These results also strongly suggest that DHECD and α-DHECD should mimic the effects of BR.

We investigated the regulatory effects of ECD, DHECD, and α-DHECD on the expression of four BR-responsive genes: *DWF4*, *CPD*, *TCH4*, and *SAUR-AC1*. The results showed that treatment with DHECD or α-DHECD significantly reduced *DWF4* mRNA expression in the *det2* mutant compared with that in the control, but ECD had no effect (Fig. 3A). DHECD and α-DHECD also significantly increased *TCH4* and *SAUR-AC1* expression in the *det2* mutant (Fig. 4C and D). *DWF4* and *CPD* are BR biosynthesis genes that are downregulated by BR [1,35], whereas *TCH4* and *SAUR-AC1* which are BR-specific expression genes are upregulated by BR [36,37]. These results suggest that DHECD and α-DHECD also regulate the expression of BR-related genes.

The phosphorylation status of the marker BIL1/BZR1 protein was performed to demonstrate the efficacy of the hydroxysteroidal compounds on BR signaling. Phosphorylation status is modulated by BR signals showed on the increase of the dephosphorylated form of BR transcriptional factor such as BRZ-INSENSITIVE-LONG HYPOCOTYL 1/BRASSINAZOLE RESISTANT 1 (BIL1/BZR1) and BRI1-EMS SUPRESSOR 1 (BES1) [26,38]. When plants were grown on Brz-containing medium, BL treatment clearly gave the high ratio of dephosphorylated BIL1/BZR1 to phosphorylated BIL1/BZR1 (Fig. 4). Among our three hydroxysteroidal compounds, DHECD and α-DHECD treatments showed the higher ratios of dephosphorylated BIL1/BZR1 to phosphorylated BIL1/BZR1 than control and ECD treatment (Fig. 4). These results indicated that DHECD and α-DHECD should mimic BR function mediated through the BR signal pathway while ECD should not. In this experiment effect of DHECD and α-DHECD were enhanced in BL deficient condition, as was the case in the recovery of hypocotyl elongation by DHECD or α-DHECD treatment in Brz-treated plants (Table 2).

Among the three hydroxysteroid compounds, we found that DHECD and α-DHECD mimicked BRs in the biological assay systems tested in this study. Differences in the effectiveness of these compounds may be a consequence of their different structures, stability or plant species used for bioassays [39]. Generally, BRs consist of four rings (A, B, C, and D) forming a steroid nucleus and a side chain attached to C-17 of D ring as is the case with cholesterol [10,40]. Studies of the structure–activity relationships of both natural and synthetic BR analogues have shown that the structures essential for high BR activity are: (1) an α-oriented hydroxyl group at either C-2 (2α-OH) or C-3 (3α-OH) in the A-ring; (2) a *trans* A/B ring junction; (3) oxygen at C-6, in the form of either a ketone or lactone in the B-ring; and (4) either a methyl or an ethyl group at C-24 in the side chain [40,41].

The BR analogues displaying high BR activity will have a structure compatible with a binding site of the BR receptor. In *Arabidopsis*, Brassinosteroid Insensitive 1 (BRI1) has been identified as the BR receptor, and consist of a leucine-rich repeat (LRR), a 70-amino-acid island domain in the N-terminal receptor-like kinase transmembrane domain, and a Ser/Thr kinase domain in the C-terminal region [42,43]. BRI1 forms a heterodimer through its LRR repeat and Ser/Thr-type transmembrane kinase domain with members of the somatic embryogenesis receptor kinase (SERK) family, such as SERK1 and SERK3 [44,45]. SERK3 was also identified as a functionally cooperative receptor of BRI1 which is referred to as BAK1 [46,47]. BR is folded between the N-terminal capping domain of SERK1 and the 70-amino-acid island domain binding pocket of BRI1. The histidine residue of SERK1 also establishes hydrogen bonds with both the 2α- and 3α-hydroxyl groups of BR [45]. Lee et al. [48] reported that 2-epicastasterone (2-epiCS) and 2,3-diepicastasterone (2,3-diepiCS), which have 2β,3α-diol and 2β,3β-diol moieties, respectively, but lack the 2α,3α-diol moiety that is assumed to be important for potent BR activity, showed 50 and 500 times less activity, respectively, than castasterone. This indicates that the 2α,3α-diol moiety in the A-ring of BR is necessary for its binding to the SERK co-receptor, which induces BR responses. However, it is important that 2-epiCS and 2,3-diepiCS are still active.

In a recent study, we showed that DHECD was synthesized by catalytic hydrogenation, which is a *cis*-reduction of the unsaturated bond on the B-ring of ECD [15]. The α-orientation of the H-8 was established by the splitting pattern and coupling constants of H-7α, H-7β and H-8 and the *cis*-relation of H-8 and H-9 in the ^{1}H NMR spectra of DHECD. The *cis*-A/B ring junction was evident from the *cis*-nature of the H-5 and the methyl group at C-10 (19-Me group) which was confirmed by the nuclear Overhauser effect (NOE) correlation between H-5 and 19-Me at C-10 [15] (Fig. S1C). Base-catalyzed epimerization of H-5 yielded α-DHECD. The a-orientation of H-5 in α-DHECD was established by the large coupling constant of H-5 and the NOE correlation between H-5 and H-3 (Fig. S1D). Both DHECD and α-DHECD have a saturated ketone group at the B-ring, which is similar to that in the active BR castasterone. The major difference between castasterone and the three hydroxysteroids considered in this study are: 1) the B/C ring junctions of DHECD and α-DHECD are *cis*, whereas those of castasterone are *trans*; and 2) ECD, DHECD, and α-DHECD have 2β,3β-dihydroxyl groups at the A-ring, whereas castasterone has 2α,3α-dihydroxyl groups. The 3D structures show that the reduction of the unsaturated bond in the B-ring of ECD to form DHECD and/or α-DHECD causes a change in the spatial relationship between the 2β,3β-diol moiety and the methyl group in the C10 position which was confirmed by NOE (Fig. S1C and D). In the case of DHECD and α-DHECD, the altered configuration differs from that of ECD (Fig. S1A). This difference in chemical structure may explain why ECD shows no BR-like activity, whereas DHECD and α-DHECD show a weak but appreciable BR-like activity. Treatment with α-DHECD caused a significant increase in hypocotyl elongation in both wild-type *Arabidopsis* treated with 0.3 μM Brz and the *det2* mutant, and had a little bigger effect than did treatment with DHECD (Table 2). The difference between DHECD and α-DHECD is in the structure of the A/B ring junction. DHECD has a *cis* A/B ring junction, whereas α-DHECD has a *trans* A/B ring junction, as occurs in BL (Figs. 1A, C and D, S1A, C and D) and castasterone. An evaluation of the biological activity of BR analogs showed that compounds possessing *trans* A/B ring junction were active than compounds possessing *cis* A/B ring junction [49,50]. However, DHECD induced the ratio of dephosphorylated BIL1/BZR1 to phosphorylated BIL1/BZR1 as same as α-DHECD (Fig. 4) indicating that they rather had similar activity on BR signaling.

Another way in which ECD, DHECD, and α-DHECD differ structurally from BR is in the side chain. The three hydroxysteroids have more hydroxyl groups in the side chain than does BL (Fig. 1). Analysis of the 3D structure of the BRI1 receptor showed that BR binds to a hydrophobic surface and maps inside the BRI1 superhelix [43]. Because the BRI1 pocket is hydrophobic, BR-like structures including too much hydroxyl groups in the side chain, such as 25-hydroxyBL, a catabolite of BL, can result in a lack of biological activity in plants [43,51,52]. This explanation is partly supported by the study by Mazorra et al. [53], who synthesized two spirostanic analogues of castasterone (MH5 and BB6) by substituting a typical BR side chain with a spiroketalic ring. This ring had a less-charged side chain than the side chain in our three hydroxysteroids, and MH5 and BB6 exhibited BR mimic effect, however they were less active than BL maybe due to the lack of appropriate distribution of hydroxyl groups in the side chain. In this context no BR-like activity for ECD and the weak activity of DHECD and α-DHECD may be attributable to the hydrophilic side chains in these analogues, especially to the hydroxyl group at 25 position. Based on the above data,

combination of 2α,3α-hydroxyl groups and a moderately charged side chain of BR could be important for binding to BRI1/BAK1 receptor complex. However, in our data DHECD and α-DHECD are still active though they have 2β,3β-OH and 25-OH. At present we cannot clearly confirm the reason why both DHECD and α-DHECD are active, but we think that it can be a good clue to design new BR mimics. For example, reducing the polarity of the side chains of DHECD and α-DHECD could be a good try to increase their BR-like activity in a future study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2017.05.003.

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