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ORIGINAL ARTICLE

Biotransformation of 6-Deoxo Type Brassinosteroids in Yeast (*Saccharomyces cerevisiae*) WAT21 Cells

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Abstract We recently demonstrated that non-plant cells, yeast WAT21, produce a steroidal plant hormone, castasterone. To understand how castasterone is generated in WAT21 cells, deuterium-labeled (26, 28-$^2H_6$) 6-deoxoteasterone, 6-deoxotyphasterol and 6-deoxocastasterone were fed to WAT21 cells, and their metabolites were analyzed by gas chromatography-mass spectrometry. When [$^2H_6$]-6-deoxoteasterone was fed, [$^2H_6$]-6-deoxo-3-dehydroteasterone and [$^2H_6$]-6-deoxotyphasterol were identified as metabolites. When [$^2H_6$]-6-deoxotyphasterol was used, [$^2H_6$]-6-deoxoteasterone and [$^2H_6$]-6-deoxo-3-dehydroteasterone and [$^2H_6$]-6-deoxocastasterone were detected. When [$^2H_6$]-6-deoxocastasterone was added, [$^2H_6$]-castasterone was identified. Taken together, a biosynthetic sequence, 6-deoxoteasterone↔6-deoxo-3-dehydroteasterone↔6-deoxotyphasterol →6-deoxocastasterone→castasterone seems to function to produce brassinosteroids in WAT21 cells. Coupled with the presence of a biosynthetic sequence, teasterone→3-dehydroteasterone↔ typhasterol →castasterone, this suggests that brassinosteroids in WAT21 cells were biosynthesized via the same kind of pathways established in plants. This study provides a clue for possible mass-production of brassinosteroids in non-plant cells, yeast WAT21 for commercial use in agriculture.

Keywords biotransformation · brassinosteroids · 6-deoxobrassinosteroids · *Saccharomyces cerevisiae*

Introduction

Brassinosteroids (BRs) are chemical signal mediators that control a variety of phenomena relevant to the growth and differentiation of plants such as stem elongation, leaf development, vascular development, photomorphogenesis stress modulation, and sink-source relationship (Bishop and Yokota, 2001; Bajguz and Tretyn, 2003). Since the first characterization of BR, brassinolide (BL) in rape (*Brassica napus*) pollen, over fifty naturally-occurring BRs have been identified from various plants ranging from higher plants (angiosperm and gymnosperm) to lower plants (fern and bryophyte), implying that BRs are present in the entire plant kingdom. We recently identified a biologically active BR, castasterone (CS), from yeast WAT21 cells (Kim et al., 2010). To the best of our knowledge, this was the first report of the detection of BRs in non-plant cells. In an effort to characterize the possible biosynthesis of BRs, feeding experiments using deuterium-labeled teasterone ([26, 28-$^2H_6$]-TE) and typhasterol ([26, 28-$^2H_6$]-TY) were carried out in WAT21 cells, resulting in the detection of a specific metabolic sequence, TE↔3-dehydroTE (3-DHT)↔TY →CS, in the cells (Lee et al., 2011). To obtain further information regarding BR biosynthesis in the yeast cells, the biotransformation of deuterium-labeled 6-deoxo type BRs, identified as biosynthetic precursors of CS, was evaluated in WAT21 cells. Herein, the presence of an alternative biosynthetic sequence for the synthesis of CS, 6-deoxoTE↔6-deoxo-3-DHT↔6-deoxoTY→6-deoxoCS →CS, in the WAT21 cells is reported.

Materials and Methods

Culture of WAT21 cells. WAT21 cells were cultured in growth medium (containing 6.7 g/L of yeast nitrogen base and 20 g/L of D-glucose). After 2 days, the cultured cells were transferred to an induction medium containing D-galactose (20 g/L).

Purification of BRs metabolites in WAT21. [26, 28-$^2H_6$]-6-deoxoTE, [26, 28-$^2H_6$]-6-deoxoTY and [26, 28-$^2H_6$]-6-deoxoCS were added to cultured WAT21 cells, and incubated for an additional 6 h. The cultured cells and media were extracted with ethyl acetate (50 mL × 3). After evaporation, the ethyl acetate

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soluble fraction was loaded onto a C18 cartridge (Waters Co., USA) eluted with aqueous methanol. The 80% methanol fraction that evidenced biological activity in the rice lamina inclination bioassay was dried *in vacuo*, dissolved in a small quantity of methanol, purified via reverse-phase high performance liquid chromatography (HPLC) (Senshu-Pak Pegasil-B ODS C18, 10 mm × 150 mm; Senshu Scientific Co., Ltd., Japan), and eluted with aqueous methanol as a mobile phase (0 to 20 min: 45%, 20 to 40 min: gradient to 100%, 40 to 60 min: 100% methanol) at a flow rate of 2.5 mL/min. Fractions were collected in 1-min intervals. The HPLC fractions corresponding to authentic BRs such as 6-deoxoTE, 6-deoxo-3-DHT, 6-deoxoTY, and 6-deoxoCS involved in the early C-6 oxidation pathway were collected.

Gas chromatography-mass spectrometry (GC-MS) analysis. Metabolites were derivatized to methaneboronate (MB), bis-methaneboronate (BMB), and methaneboronate-trimethylsilyl ether (MB-TMSi), and subsequently analyzed via capillary GC-MS: a Hewlett-Packard (USA) 5973 mass spectrometer (Electron impact ionization, 70 electron voltage) coupled to a 6890 GC 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, raised to 280°C at 40°C/min, then maintained at 280°C. Helium was employed as the carrier gas at 1 mL/min, and the samples were introduced via on-column injection mode.

Results and Discussion

[26, 28-$^2H_6$]-6-deoxoTE, [26, 28-$^2H_6$]-6-deoxoTY and [26, 28-$^2H_6$]-6-deoxoCS were individually added to WAT21 cells as the substrates, and incubated for an additional 6 h. Metabolites in the cultured cells and media were extracted with ethyl acetate. The obtained ethyl acetate soluble fractions were purified via a C18 cartridge and subsequently via a reverse-phase HPLC. The HPLC fractions corresponding to authentic BRs, such as 6-deoxoTE, 6-deoxo-3-DHT, 6-deoxoTY, and 6-deoxoCS were collected, derivatized to MB, BMB, or MB-TMSi, and then analyzed via capillary GC-MS/selected ion monitoring (SIM).

When [$^2H_6$]-6-deoxoTE was used as a substrate, MB-TMSi on an active principle in HPLC fractions 51 to 53, which corresponds to synthetic 6-deoxoTY under identical HPLC conditions, yielded a molecular ion at *m/z* 536 and prominent ions at *m/z* 521, 479, 446, 431, 305, 215, and 161. These were identical to those derived from authentic [$^2H_6$]-6-deoxoTY MB-TMSi ether (Table 1). The retention time on GC of the MB-TMSi of the active principle was identical to that of [$^2H_6$]-6-deoxoTY MB-TMSi. Therefore, the metabolite was identified as [$^2H_6$]-6-deoxoTY. MB of a compound in the same HPLC fractions exhibited a molecular ion at *m/z* 462 and characteristic ions at *m/z* 447, 385, 301, 246, 231, and 161 on a GC retention time identical to that of [$^2H_6$]-6-deoxo-3-DHT MB. The compound was thus identified as [$^2H_6$]-6-deoxo-3-DHT. Collectively, our results showed that the conversion of [$^2H_6$]-6-deoxoTE to [$^2H_6$]-6-deoxoTY intermediated by [$^2H_6$]-6-deoxo-3-DHT occurred in the yeast cells. Enzyme activity for C-3 epimerization of [$^2H_6$]-6-deoxoTE to [$^2H_6$]-6-deoxoTY intermediated by [$^2H_6$]-6-deoxo-3-DHT was 184 pg · g cell^{-1} · min^{-1} in WAT21.

When [$^2H_6$]-6-deoxoTY was fed to the yeast cells, the MB-TMSi of a compound in HPLC fractions 51 to 53 exhibited a molecular ion at *m/z* 536 and characteristic ions at *m/z* 521, 479, 446, 431, 305, 215, and 161. These were identical to those of authentic [$^2H_6$]-6-deoxoTE MB-TMSi ether. Additionally, the GC retention time of the MB-TMSi of the compound was found to be identical to that of [$^2H_6$]-6-deoxoTE MB-TMSi; thus the compound was definitively identified as [$^2H_6$]-6-deoxoTE. The MB of an active principle in the same HPLC fractions yielded an identical mass spectrum at *m/z* 462 [M+], 246, 231, and 161 to that derived from [$^2H_6$]-6-deoxo-3-DHT MB at an identical GC retention time, thereby identifying the active principle as [$^2H_6$]-deoxo-3-DHT.

Table 1 GC-MS data for metabolites of [$^2H_6$]-6-deoxoTE, [$^2H_6$]-6-deoxo-3DHT and [$^2H_6$]-6-deoxoTY fed to WAT21 yeast cells

Compound		Rt* on GC	Prominent ions (relative intensity %)
Substrate	Product		
[$^2H_6$]-6-deoxoTE	[$^2H_6$]-6-deoxo-3-DHT**	13.91	462 (M^+, 47), 447 (7), 385 (5), 301 (9), 246 (12), 231 (100), 161 (46)
	[$^2H_6$]-6-deoxoTY****	12.01	536 (M^+, 25), 521 (4), 479 (3), 446 (46), 431 (48), 305 (13), 215 (100), 161(28)
[$^2H_6$]-6-deoxoTY	[$^2H_6$]-6-deoxo-3-DHT**	13.91	462 (M^+, 50), 447 (8), 385 (6), 301 (9), 246 (14), 231 (100), 161 (47)
	[$^2H_6$]-6-deoxoTE****	13.78	536 (M^+, 49), 521 (47), 479 (16), 446 (19), 431 (27), 305 (28), 215 (100), 161 (41)
	[$^2H_6$]-6-deoxoCS***	14.22	504 (M^+, 41), 489 (16), 273 (100), 161 (35)
[$^2H_6$]-6-deoxoCS	[$^2H_6$]-CS***	21.79	518(M^+,81), 441(6), 358(35), 327(13), 287(34), 161(100)
Authentic	[$^2H_6$]-6-deoxoTE****	13.78	536 (M^+, 50), 521 (46), 479 (17), 446 (18), 431 (29), 305 (28), 215 (100), 161 (42)
Authentic	[$^2H_6$]-6-deoxo-3-DHT**	13.91	462 (M^+, 49), 447 (6), 385 (4), 301 (8), 246 (12), 231 (100), 161 (45)
Authentic	[$^2H_6$]-6-deoxoTY****	12.01	536 (M^+, 23), 521 (3), 479 (2), 446 (47), 431 (48), 305 (12), 215 (100), 161(29)
Authentic	[$^2H_6$]-6-deoxoCS***	14.22	504 (M^+, 40), 489 (14), 273 (100), 161 (34)
Authentic	[$^2H_6$]-CS***	21.79	518(M^+,80), 441(7), 358(33), 327(12), 287(32), 161(100)

*Rt : retention time (min)
**The sample was derivatized as a methaneboronate (MB)
***The sample was derivatized as a bismethaneboronate (BMB)
****The sample was derivatized as a methaneboronatetrimethylsilyl ether (MB-TMSi)

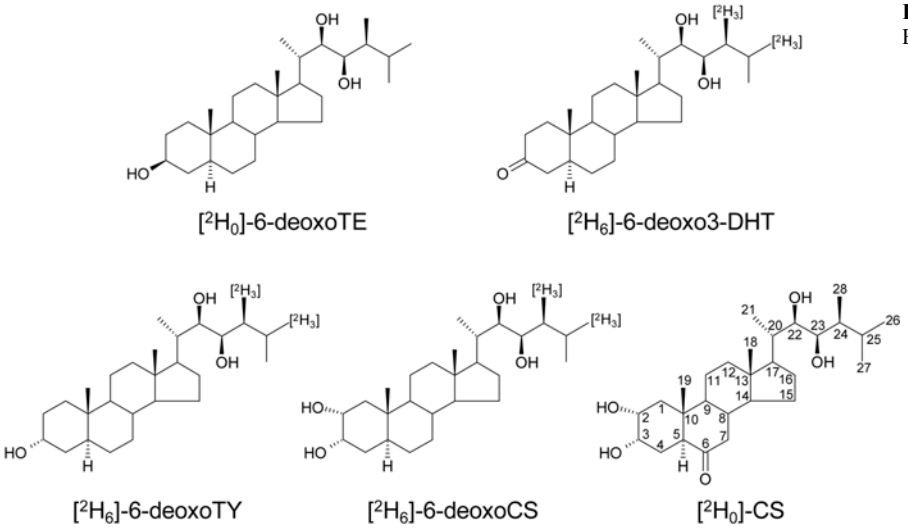

Fig. 1 Chemical structure of deuterium-labeled BRs.

The BMB of an active compound in HPLC fractions 40 to 42 exhibited a molecular ion at *m/z* 504 and characteristic ions at *m/z* 489, 273 and 161. Thus, the mass spectrum and GC retention time of BMB of the compound were equal to those of [$^2H_6$]-6-deoxoCS BMB, and the compound was identified as [$^2H_6$]-6-deoxoCS. Consequently, four metabolites of [$^2H_6$]-6-deoxoTY, [$^2H_6$]-6-deoxoTE, [$^2H_6$]-6-deoxo-3-DHT and [$^2H_6$]-6-deoxoCS were identified, thus demonstrating a reversible C-3 epimerization of [$^2H_6$]-6-deoxoTE to [$^2H_6$]-6-deoxoTY via [$^2H_6$]-6-deoxo-3-DHT and 2α-hydroxylation of [$^2H_6$]-6-deoxoTY to [$^2H_6$]-6-deoxoCS in the yeast cells. Enzyme activity for 2α-hydroxylation of [$^2H_6$]-6-deoxoTY to [$^2H_6$]-6-deoxoCS was 80 $pg \cdot g\ cell^{-1} \cdot min^{-1}$ in the cell.

When [$^2H_6$]-6-deoxoCS was added to the medium, the BMB of a compound in HPLC fraction 19 to 21 exhibited a molecular ion at *m/z* 518 and characteristic ions at *m/z* 441, 358, 327, 287 and 161. These mass ions and retention time on GC were equal to those of authentic [$^2H_6$]-CS BMB, showing that the compound was [$^2H_6$]-CS and that the C-6 oxidation of [$^2H_6$]-6-deoxoCS to [$^2H_6$]-CS occurs in the WAT21 cells. Enzyme activity for C-6 oxidation of [$^2H_6$]-6-deoxoCS to [$^2H_6$]-CS was 11 $pg{\cdot}g\ cell^{-1}{\cdot}min^{-1}$ in WAT21. Along with the results obtained from metabolic studies of [$^2H_6$]-6-deoxoTE and [$^2H_6$]-6-deoxoTY, this finding provides concrete evidence for the biotransformation of [$^2H_6$]-6-deoxoTE ↔[$^2H_6$]-6-deoxo-3-DHT↔[$^2H_6$]-6-deoxoTY→[$^2H_6$]-6-deoxoCS [$^2H_6$]-CS in the yeast cells.

The results of the present study demonstrated that WAT21 yeast cells have enzyme activities for the conversion of [$^2H_6$]-6-deoxoTE to [$^2H_6$]-CS via [$^2H_6$]-6-deoxo-3-DHT, [$^2H_6$]-6-deoxoTY and [$^2H_6$]-6-deoxoCS, strongly suggesting that a biosynthetic sequence, 6-deoxoTE↔6-deoxo-3-DHT↔6-deoxoTY→6-deoxoCS→CS, underlies the generation of CS in the yeast cells. Along with our previous findings of the presence of a biosynthetic sequence, TE ↔3-DHT↔TY→CS, in the WAT21 cells, this finding shows that CS, which is a known biologically active BR in plants, can be

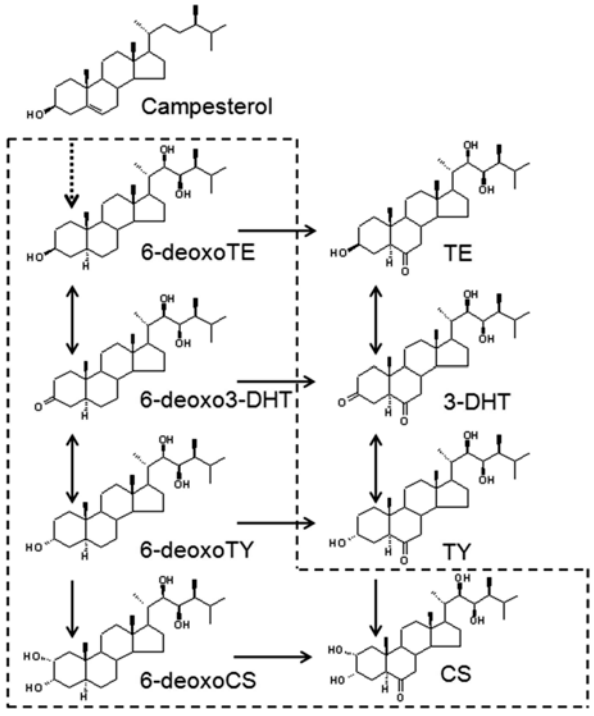

Fig. 2 Biosynthetic pathway for BRs identified in WAT21 yeast cells. The biosynthetic sequence in the box was established. The dotted and solid arrows indicate multiple and single reactions, respectively

biosynthesized by two parallel biosynthetic pathways through 6-deoxo and 6-oxo BRs in yeast cells. 6-Oxo BRs have been generally thought to be biosynthesized from campesterol (CR) through the early C-6 oxidation pathway (Sakurai et al., 1999; Buchnan et al., 2000; Bishop and Yokota, 2001). However, functional studies of the cytochrome P450s (CYP) involved in BRs biosynthesis, including CYP90A1, 90B1, 90C1, and 90D1, as well as metabolic studies using biosynthetically upstream

intermediates such as 6-oxocampestanol (6-oxoCN) and cathasterone (CT), have revealed that the conversion of 6-oxoCN to TE via CT does not occur in plants (Joo et al., 2002). Therefore, the 6-oxo BRs in plants are thought to be biosynthesized via C-6 oxidation from 6-deoxo BRs, which are mediated by CYP85A1 and A2 (Kwon et al., 2005). In the present study, WAT21 was demonstrated to have enzyme activity for the C-6 oxidation of 6-deoxoCS to CS, thereby implying that 6-oxo BRs could be biosynthesized from 6-deoxo BRs in yeast cells. To the best of our knowledge, yeast has only four CYPs (NCP1 as NADP-CYP reductase, ERG5 as C-22 sterol desaturase, ERG11 lanosterol 14α-demethylase, and DAP1 as a damage response protein) in its genome (Turi and Loper, 1992; Kelly et al., 1995; Lees et al., 1995; Mallory et al., 2005). This suggests that the C-6 oxidation of 6-deoxo BRs, at least 6-deoxoCS, could be catalyzed by a non-CYP monooxygenase in yeast. Therefore, the C-6 oxidation of 6-deoxo BRs by CYPs is believed to be a reaction acquired after evolution to plants.

Enzyme activities involved in C-3 epimerization for 6-deoxoTE to 6-deoxoTY and 2α-hydroxylation of 6-deoxoTY to 6-deoxoCS in WAT21 are comparable to those in plants (unpublished data). However, enzyme activity for C-6 oxidation in the yeast cell is quite lower (ca. 110^{-1}–120^{-1}) than that in plants (unpublished data). Previously, Arabidopsis BR 6-oxidases, CYP85A1 and A2 were heterologously expressed in WAT21 (Kim et al., 2005). The conversion rates of 6-deoxoCS to CS by CYP85A1 and A2 were approximately 50 and 80%, respectively. Therefore, the low activity for native BR 6-oxidase in WAT21 can be overcome by insertion of Arabidopsis CYP85A1 and A2.

WAT11 and WAT21 are yeast cell lines, into which Arabidopsis NADPH P450 reductase 1 and 2 are inserted (Urban et al., 2005). Many plant CYPs including CYP85A1 and A2 in BRs biosynthesis were heterologously expressed in WAT11 or WAT21 to determine their functions in plants (Kim et al., 2005; Duan and Schuler, 2006). In the present study, WAT21 was found to possess enzyme activities for BR biosynthesis, suggesting that WAT11 may also contain enzyme activates for BR biosynthesis. For verification, metabolic studies using intermediates involved in BR biosynthesis are presently underway in WAT11.

BRs are regarded as promising candidates for a broad range of commercial agricultural applications. However, the high cost currently required for the preparation of BRs for such applications remains a problem that must be solved before the commercial use of BRs in agriculture can be realized. In an effort to overcome this problem, the possible production of BRs in microbial organisms was evaluated, which ultimately resulted in the identification of a biologically active BR, CS, in the WAT21 yeast strain. Additionally, some possible biosynthetic sequences that synthesize BRs in the yeast cells were established, thereby providing important hints as to the manner in which BRs can be produced on a massive scale with low cost in non-plant cells and most probably in yeast cells.

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