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Biosynthesis of Brassinosteroids in a Yeast (*Saccharomyces cerevisiae*) Cell, WAT21: Conversion of Teasterone to Castasterone

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Brassinosteroids (BRs) are endogenous steroidal plant hormones that regulate a variety of growth and development functions in plants at very low concentrations (> 10^{-9} M).[1] BRs are, therefore, considered promising candidates for commercial agricultural applications.[2-6] However, the high cost inherent to the preparation of BRs for use in agricultural applications remains a problem that must be resolved before BRs can be used in commercial agricultural applications. In order to overcome this problem, we have evaluated the possible production of BRs in microbial organisms, resulting in the identification of an active BR, castasterone (CS, Fig. 1),[7] in the yeast (*Saccharomyces cerevisiae*) strain, WAT21. This prompted us to conduct an investigation into the synthesis of CS in the yeast cells.

In plants, CS has been shown to be biosynthesized from campesterol (CR), which harbors the same carbon skeleton as that of CS *via* two parallel biosynthetic pathways − namely, the early and late C-6 oxidation pathways.[8] In an effort to assess the possible presence for the early C-6 oxidation pathway to generate CS in the WAT 21 yeast strain, cultured cells and media of WAT21 were extracted with ethyl acetate (50 mL × 3). After purification by a reversed phase HPLC, the HPLC fractions corresponding to authentic BRs such as TE, 3-dehyroTE, TY, and CS involved in the early C-6 oxidation pathway were collected, derivatized to methaneboronate (MB), bismethaneboronate (BMB), or methaneboronate-trimethylsilyl ether (MB-TMSi), and subsequently analyzed *via* capillary GC-MS.

When [$^2H_6$]-TE was added to the media, MB-TMSi on an active principle in HPLC fractions 32 and 33, which correspond to synthetic TY under identical HPLC conditions yielded a molecular ion at *m*/*z* 550 and prominent ions at *m*/*z* 535, 521, and 161; these are identical to those derived from authentic [$^2H_6$]-TY MB-TMSi ether (Table 1). The retention time on GC of the MB-TMSi of the active principle is precisely identical to that of [$^2H_6$]-TY MB-TMSi. Therefore, the metabolite of [$^2H_6$]-TE was identified as [$^2H_6$]-TY. The MB of a compound in HPLC fraction 38 evidenced a molecular ion at *m*/*z* 476 and characteristic ions at *m*/*z* 357, 316, 161 at a GC retention time (31.58 min) identical to those of [$^2H_6$]-3-dehydroTE MB. The compound was thus identified as [$^2H_6$]-3-dehydroTE. Collectively, our results showed definitively that the conversion of [$^2H_6$]-TE to [$^2H_6$]-TY intermediated by [$^2H_6$]-3-dehydroTE had occurred in the yeast cells.

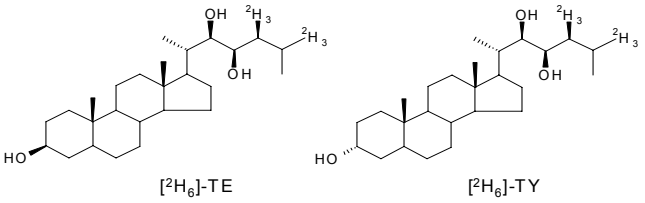

Figure 2. Structure of [$^2H_6$]-TE and [$^2H_6$]-TY fed to a yeast cell, WAT21.

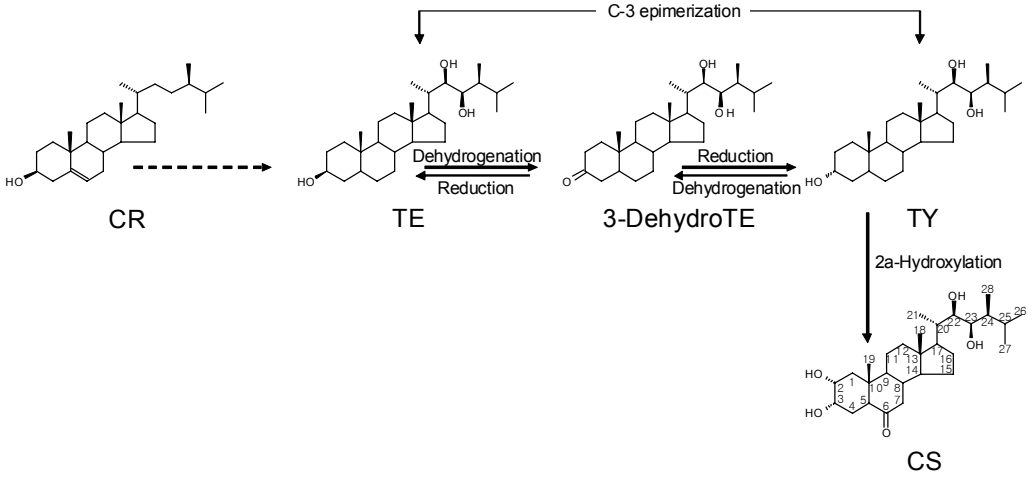

Figure 1. A biosynthetic pathway for BRs established in a yeast cell, WAT21. The dotted arrow indicates multiple biosynthetic steps. The solid arrows indicate a single biosynthetic step.

Table 1. HPLC and GC-MS data for enzyme products for [$^2H_6$]-TE and [$^2H_6$]-TY in a yeast cell, WAT21

Compound		Rt† on HPLC	Rt† on GC	Prominent ions (relative intensity, %)
Substrate	Product			
[$^2H_6$]-TE	[$^2H_6$]-3-dehydroTE[a]	38	31.58	476(M+, 63), 399(7), 357(5), 316(21), 298(10), 287(11), 245(11), 161(100)
	[$^2H_6$]-TY[b]	37 - 38	26.90	550(M+, 100), 535 (81), 521(55), 460(72), 306(10), 161(60)
[$^2H_6$]-TY	[$^2H_6$]-3-dehydroTE[a]	38	31.58	476(M+, 60), 399(10), 357(5), 316(19), 298(11), 287(11), 245(10), 161(100)
	[$^2H_6$]-TE[b]	32 - 33	32.14	550(M+, 29), 535 (53), 521(100), 460 (5), 306 (8), 161(39)
	[$^2H_6$]-CS[c]	19 - 20	31.17	518(M+, 80), 441(5), 389(11), 358(33), 327(12), 287(32), 16(100)
Authentic [$^2H_6$]-TE[b]		32 - 33	32.14	550(M+, 30), 535(52), 521(100), 460(5), 306(7), 161(40)
Authentic [$^2H_6$]-3-dehydroTE[a]		38	31.58	476(M+, 63), 399(7), 357(5), 316(21), 298(10), 287(11), 245(11), 161(100)
Authentic [$^2H_6$]-TY[b]		37-38	26.90	550(M+, 100), 535(80), 521(53), 460(73), 306(11), 161(59)
Authentic [$^2H_6$]-CS[c]		19-20	31.17	518(M+, 78), 441(4), 389(12), 358(32), 327(10), 287(34), 161(100)

[a]MB (bismethaneboronate). [b]MB-TMS (methaneboronate-trimethylsilyl ether). [c]BMB (bismethaneboronate). †Retention time (min).

Table 2. Identification of CR in a yeast cell, WAT21, by a capillary GC-MS analysis

Compound*	Rt† on GC	Prominent ions (relative intensity, %)
CR in WAT21	13.78	472(18), 457(6), 382(47), 367(23), 343(58), 315(1), 261(12), 255(18), 227(2), 213(13), 129(100)
Authentic CR	13.78	472(16), 457(6), 382(43), 367(21), 343(56), 315(3), 261(12), 255(19), 227(2), 213(13), 129(100)

*Compound was analyzed as a bismethaneboronate (BMB). †Retention time (min).

When [$^2H_6$]-TY was fed to the yeast cells, the MB-TMSi of a compound in HPLC fractions 37 and 38 evidenced a molecular ion at *m*/*z* 550 and characteristic ions at *m*/*z* 535, 521, 161; these were identical to those of authentic [$^2H_6$]-TE MB-TMSi ether. Additionally, the GC retention time of the MB-TMSi of the compound was found to be identical to that of [$^2H_6$]-TE MB-TMSi, thus definitively identifying the compound as [$^2H_6$]-TE. The MB of an active principle in HPLC fraction 38 yielded an identical mass spectrum at *m*/*z* 476 [M+], 357, 316, 161 to those derived from [$^2H_6$]-3-dehydroTE MB at an identical GC retention time, thus demonstrating that the active principle is [$^2H_6$]-3-dehydroTE. The BMB of an active compound in HPLC fraction 19 and 20 showed a molecular ion at *m*/*z* 518 and characteristic ions at *m*/*z* 358, 327, 287 and 161. Thus, the mass spectrum and GC retention time of BMB of the compound were precisely equal to those of [$^2H_6$]-CS BMB, thus identifying the compound as [$^2H_6$]-CS. Consequently, three metabolites of [$^2H_6$]-TY, [$^2H_6$]-TE, [$^2H_6$]-3-dehydroTE and [$^2H_6$]-CS were identified, thus demonstrating a reversible C-3 epimerization of [$^2H_6$]-TE to [$^2H_6$]-TY *via* [$^2H_6$]-3-dehydroTE, [$^2H_6$]-TE ↔ [$^2H_6$]-3-dehydroTE ↔ [$^2H_6$]-TY, and 2α-hydroxylation of [$^2H_6$]-TY to [$^2H_6$]-CS in the yeast cells.

The aforementioned feeding experiments using deuterium-labeled BRs strongly indicate that a biosynthetic sequence, TE ↔ 3-dehydroTE ↔ TY → CS is operant in WAT21 cells. In plants, these BRs are biosynthesized from CR,[9] which is not commonly detected in yeast (*Saccharomyces cerevisiae*) cells. The presence of CR in WAT21 cells was evaluated. To that end, the cultured cells and medium were extracted with *n*-hexane (50 mL × 3). The *n*-hexane soluble fraction was saponified for 1 h with 5% KOH in 80% ethanol at 70 °C. The unsaponified lipids were extracted with *n*-hexane and purified *via* reverse-phase HPLC (Pergasil-B ODS, 10 × 150 mm). The HPLC fraction 16 was derivatized as a TMSi ether, and analyzed *via* capillary GC-MS, as described above. As summarized in Table 2, the mass spectrum and GC retention time obtained herein were identical to those of CR TMSi ether, thereby demonstrating the presence of CR in the yeast cells.

Throughout the entire plant kingdom − from green alga to vascular plants − CS is biosynthesized from CR *via* two parallel biosynthetic pathways: namely, the early and late C-6 oxidation pathways.[8] The established biosynthetic sequence, TE ↔ 3-dehydroTE ↔ TY → CS, is a partial biosynthetic pathway that is involved in the early C-6 oxidation pathway, which produces CS. Coupled with the presence of CR, this strongly implies that the early C-6 oxidation pathway contributes to the generation of CS in WAT21 yeast cells. In *Arabidopsis thaliana*, upstream biosynthetic reactions from CR to campesterol (CN), 6-oxoCN to cathasterone (CT) and CT to TE in the early C-6 oxidation pathway are known to be catalyzed by DET2, DWARF4, and CPD, respectively.[10-12] The enzymes (genes) responsible for C-3 epimerization from TE to TY *via* 3-dehydroTE and 2α-hydroxylation of TY to CS have yet to be clearly characterized. The characterization of C3-epimerase and 2α-hydroxylase in yeast cells are expected to provide important insights into the manner in which plant enzymes (genes) mediate the reactions relevant to BR biosynthesis.

The presence of a partial biosynthetic sequence, TE ↔ 3-dehydroTE ↔ TY → CS, in WAT21 indicates that the early C-6 oxidation pathway for CS is a steroid biosynthesis that is well-conserved during plant evolution. In fact, C3-epimerization and 2α-hydroxylation in the late C-6 oxidation pathway for CS, 6-deoxoTE ↔ 6-deoxo-3-dehydroTE ↔ 6-deoxoTY → 6-deoxoCS, is basically the same reaction set as that observed in the early C-6 oxidation pathway; this also implies that the late C-6 oxidation pathway for CS simultaneously operates in the yeast cells.

Previously, we have shown that WAT21 has no enzyme acti-

vity catalyzing the conversion of CS to brassinolide (BL), which evidences more profound biological activity than that of CS.[13] However, heterologously expressed a cytochrome P450 (CYP85 A2) in WAT21, which is designated as CYP85A2/V60/WAT21, successfully mediates the lactonation of CS to BL.[14] This implies that the genetic engineering of BR biosynthetic genes in yeast cells appears to be a promising procedure for the production of agriculturally valuable BRs in industry. For this reason, yeast cell lines containing multiple genes for BR biosynthesis are currently under construction.

Experimental Section

Culture of WAT21 Cells. WAT21 cells were cultured in growth media (a minimal nutrition media containing 6.7 g/L of a 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). 12 hours later, deuterium-labeled teasterone ([26, 28^{-2}H$_6$]-TE) and typhasterol ([26, 28^{-2}H$_6$]-TY) were added, and incubated for an additional 6 hours.

Identification of BRs Metabolized in WAT21 Cells. The cultured cells and media were extracted with ethyl acetate (50 mL × 3). After evaporation, the ethyl acetate soluble fraction was loaded onto a C18 cartridge (Waters Co.) 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, and was purified *via* reverse-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% methanol) at a flow rate of 2.5 mL/min. Fractions were collected every minute. The HPLC fractions corresponding to authentic BRs such as TE, 3-dehyroTE, TY, and CS involved in the early C-6 oxidation pathway were collected, derivatized to MB, BMB, or MB-TMSi, and subsequently analyzed *via* 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, raised to 280 °C at a rate of 40 °C min^{-1}, 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.

Identification of CR in WAT21 Cells. The cultured cells and medium were extracted with *n*-hexane (50 mL × 3). The *n*-hexane soluble fraction was dried in vacuo, and saponified for 1 h with 5% KOH in 80% ethanol at 70 °C. The unsaponified lipids were extracted with *n*-hexane (50 mL × 3), and purified *via* reverse-phase HPLC (Pergasil-B ODS, 10 × 150 mm) using 100% methanol as a mobile phase at a flow rate of 2.5 mL/min. Fractions were collected every minute. HPLC fraction 16 was derivatized as a TMSi ether, and analyzed *via* capillary GC-MS, as described above.

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