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Identification and Biosynthesis of Cholest-4-en-3-one and 6-Oxocholetanol in Young Tomato Plants

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Polyhydroxylated plant steroids, collectively referred to as the brassinosteroids (BRs), are a new class of plant hormones that regulate a variety of phenomena in plant growth and development.[1,2] Thus far, more than 40 BRs have been identified throughout the entirety of the plant kingdom.[3,4] Naturally-occurring BRs can be classified as C_{27}-, C_{28}- and C_{29}-BRs on the basis of their carbon skeletons. The structural similarity between BRs and phytosterols suggests that BRs may be biosynthesized from phytosterols, which have the same carbon skeletons as BRs.[5,6] In fact, bio-organic feeding experiments and molecular genetic studies have revealed that biologically active C_{28}-BRs, castasterone (Fig. 1, CS) and brassinolide, are biosynthesized from campesterol (CR) by two parallel biosynthetic pathways designated the early and late C6-oxidation pathway--in many plants.[7] Recently, we showed that young tomato plants exploit a novel biosynthetic pathway to generate a C_{27}-BR, 28-norCS from cholesterol (CHR).[7] The pathway for C_{27}-BRs biosynthesis in the tomato plants was basically identical to that of the late C6-oxidation pathway for C_{28}-BRs biosynthesis, thus indicating that the late C6-oxidation pathway for C_{27}-BRs biosynthesis is functional in the plants. This finding compelled us to assess the possible operation of the early C6-oxidation pathway for C_{27}-BRs biosynthesis in the tomato plants. Toward this end, we attempted to identify biosynthetic precursors in the early C6-oxidation pathway for C_{27}-BRs biosynthesis in young tomato plants, and characterize their metabolism.

The unsaponified lipid obtained from young tomato plants was purified with an SiO_2 and a Sep-Pak C18 column chromatography. After reversed-phase HPLC (Senshu-Pak Pegasil-B, 4.6 × 250 mm), the fractions corresponding to authentic 6-oxoCHN (18 - 19 min) and cholest-4-en-3-one (37 - 39 min) were collected, and then analyzed *via* capillary GC-MS.[3]

After 30 minutes of treatment of *N*-methyl-*N*-TMS-trifluroacetamide at 70 °C, trimethylsillylic (TMSi) ether of a compound in HPLC fractions 18 and 19 yielded a molecular ion at *m*/*z* 474 and prominent ions at *m*/*z* 459, 445, 384, 221 and 159 at the same GC retention time (19.51 min) as that of authentic 6-oxoCHN TMSi ether (Table 1). Therefore, the compound was identified as 6-oxoCHN. Without derivatization, a compound in HPLC fraction 37 - 39 evidenced a molecular ion at *m*/*z* 384 and characteristic ions at *m*/*z* 342, 261, 229 and 124 with identical retention times on GC to that of synthetic cholest-4-en-3-one; this finding confirmed the identification of the compound as cholest-4-en-3-one. Consequently, cholest-4-en-3-one and 6-oxoCHN were detected in the tomato plants and identified as potent intermediates involved in the early C6-oxidation pathway for C_{27}-BRs.

In order to confirm the involvement of cholest-4-en-3-one and 6-oxoCHN in the early C6-oxidation pathway for C_{27}-BRs, *in vitro* enzymatic conversions of related sterols such as CHR, cholest-4-en-3-one, and CHN were conducted. The reactions were initiated *via* the addition of substrates (CHR, cholest-4-en-3-one and CHN), followed by 30 min of incubation at 37 °C. The metabolites of the enzyme reactions were extracted with ethyl acetate, and purified by a Sep-Pak C18 cartridge and a reversed-phase HPLC. Finally, the enzyme products were

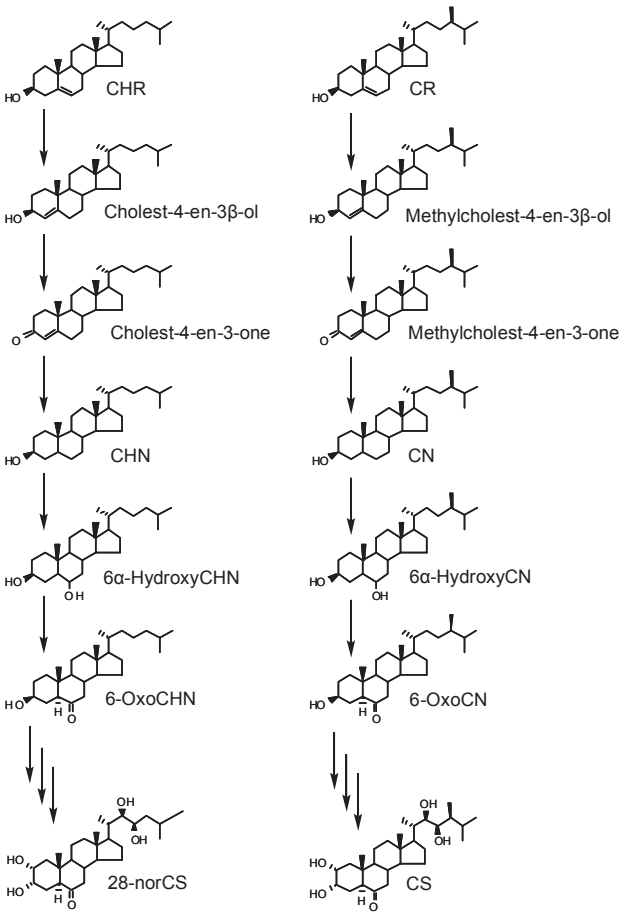

Figure 1. The upstream biosynthetic pathway for the early C6-oxidation pathway for C_{27}- and C_{28}-BRs biosynthesis.

Table 1. GC-MS data for 6-oxoCHN and cholest-4-en-3-one in young tomato plants.

Compound	Rt[a] (min) on GC	Prominent ions (relative intensity, %)
Tomato 6-oxoCHN[b]	19.51	474 (M+, 19), 459 (51), 445 (100), 384 (4), 159 (8)
Tomato cholest-4-en-3-one	13.60	384 (M+, 58), 369 (8), 342 (22), 261 (26), 229 (42), 124 (100)
Authentic 6-oxoCHN[b]	19.51	474 (M+, 18), 459 (48), 445 (100), 384 (6), 159 (10)
Authentic cholest-4-en-3-one	13.60	384 (M+, 60), 369 (8), 342 (24), 261 (24), 229 (46), 124 (100)

[a]Retention time. [b]The samples were analyzed as a derivative of TMSi ether.

Table 2. Enzymatic conversion of CHR, cholest-4-en-3-one and CHN in young tomato plants.

Substrate	Product	Prominent ions (relative intensity, %)
CHR	Cholest-4-en-3-one	384 (M+, 61), 369 (9), 342 (22), 261 (25), 229 (40), 124 (100)
	CHN[a]	460 (M+, 54), 445 (73), 370 (28), 355 (43), 305 (33), 215 (100)
Cholest-4-en-3-one	CHN[a]	460 (M+, 73), 445 (90), 370 (46), 355 (56), 305 (35), 215 (100)
CHN	6-OxoCHN[a]	474 (M+, 19), 459 (51), 445 (100), 384 (4), 159 (8)

[a]The samples were analyzed as a derivative of TMSi ether.

analyzed *via* capillary GC-MS/SIM after suitable derivatization.

As summarized in Table 2, when CHR was added as a substrate, two products were identified: cholest-4-en-3-one and CHN. When cholest-4-en-3-one was used, CHN was generated as a product. Thus, the presence of a biosynthetic sequence, CHR → cholest-4-en-3-one → CHN was demonstrated. When CHN was utilized as a substrate, 6-oxoCHN was detected as an enzyme product. Therefore, the conversion of CHN to 6-oxoCHN was confirmed. Taken together, the presence of a partial biosynthetic sequence for the early C6-oxidation pathway, CHR → cholest-4-en-3-one → CHN → 6-oxoCHN, was confirmed in the tomato plants.

The conversion of CHN to 6-oxoCHN involves the incorporation of an oxygen atom at the C-6 of CHN.[7] In the late C6-oxidation pathway for C_{27}-BRs biosynthesis in the tomato, the same C6-oxidation from 6-deoxo-28-norCS to 28-norCS has been shown to be catalyzed by a cytochrome P450, designated as CYP85,[7] thereby suggesting that the conversion of CHN to 6-oxoCHN can also be catalyzed by CYP85 in the tomato. To evaluate this possibility, enzyme solutions were prepared from a wild-type (Alisa) and a dwarf mutant (*dx*) with a defective CYP85 gene, and the conversion of CHN to 6-oxoCHN was assessed with the enzyme solutions using the same enzyme assay as described above. As shown in Figure 2, both enzyme solutions prepared from the wild-type and *dx* mutant successfully mediated the 6-oxidation of CHN to 6-oxoCHN, thereby indicating that the 6-oxidation of CHN to 6-oxoCHN is not catalyzed by CYP85 in the tomato plant.

This study is the first to demonstrate that two sterols--cholest-4-en-3-one and 6-oxoCHN--are contained in young tomato plants. Cholest-4-en-3-one and 6-oxoCHN are the counterparts of campest-4-en-3-one and 6-oxoCN in the early C6-oxidation pathway for C_{28}-BRs biosynthesis, respectively (Fig. 1). This suggests that cholest-4-en-3-one and 6-oxoCHN are biosynthesized and metabolized in a manner similar to that of campest-4-en-3-one and 6-oxoCN in C_{28}-BRs biosynthesis in plants. Therefore, cholest-4-en-3-one is believed to be generated from

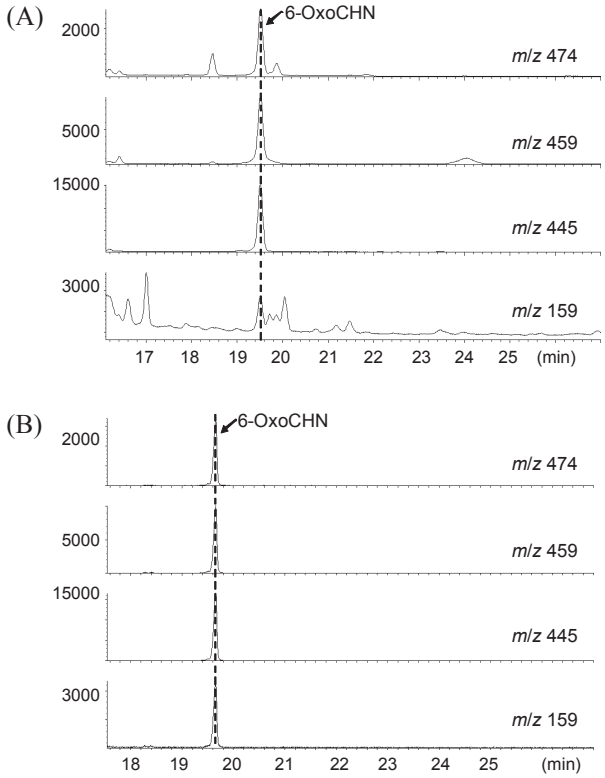

Figure 2. Enzymatic conversion of CHN to 6-oxoCHN in wild-type (A) and *dx* mutant (B) of young tomato plants.

CHR *via* cholest-4-en-3β-ol, and converted into CHN *via* 5α-cholestan-3-one. CHN is believed to be metabolized into 6-oxoCHN *via* 6α-hydroxyCHN in tomato plants.

In vitro enzyme assay using tomato *dx* mutant shows that CYP85 is not responsible for the 6-oxidation of CHN to 6-oxoCHN in tomato. In *Arabidopsis thaliana*, two orthologs of tomato CYP85, designated as CYP85A1 and CYP85A2 have been identified.[3] Molecular genetic and biochemical analysis of CYP85A1 and CYP85A2 revealed that CYP85A1 is a BR

6-oxidase, and CYP85A2 is a bi-functional enzyme whose activity is not only BR 6-oxidase but also brassinolide (BL) synthase which catalyze the conversion of CS to BL, the last step of C28-BRs biosynthesis. Recently, the same bi-functional enzyme as CYP85A2, designated as CYP85A3, has been characterized in tomato fruit.[8] We found that both CYP85A1 and CYP85A2 are not responsible for the 6-oxidation of CHN to 6-oxoCHN in *Arabidopsis* (data published elsewhere), suggesting that CYP85A3 dose not catalyze the 6-oxidation in tomato. However, the final conclusion should be waited until the precise metabolic study will be carried out with tomato *cyp85a3* mutant.

In a previous study, we demonstrated the presence of the late C6-oxidation pathway for C_{27}-BRs in tomato plants.[7] In this study, the presence of a partial biosynthetic sequence for the early C6-oxidation pathway for C_{27}-BRs, CHR → cholest-4-en-3-one → CHN → 6-oxoCHN, was confirmed in the plants. Although the downstream biosynthetic sequence from 6-oxoCHN to 28-norCS has yet to be clearly elucidated, the results of this study indicate that both the early and late C6-oxidation pathway for C_{27}-BRs biosynthesis can generate 28-norCS in tomato plants. In the aid for *S*-adenosyl-methionine and NADPH, 28-norCS is methylated at C-24 to synthesize active C_{28}-BR, CS in tomato plants.[7] Considering that CS is more biologically active than 28-norCS, the early and late C6-oxidation pathway for C_{27}-BRs biosynthesis can be concluded to be alternative routes for the synthesis of biologically active C_{28}-BR, CS in the plants.

Experimental Section

Isolation of 6-oxoCHN and cholest-4-en-3-one in young tomato plants. Young tomato plants (100 g) were homogenized with 80% methanol (1 L), followed by chloroform (1 L). The chloroform soluble fraction was dried in vacuo, and partitioned between *n*-hexane (500 mL) and 70% methanol (500 mL). The obtained *n*-hexane soluble fraction was concentrated in dryness and unsaponified for 2 hours with 5% KOH in 70% ethanol. After extraction with *n*-hexane, the unsaponified lipids (100 mg) were purified with an SiO_2 (20 g, Merck) column eluted with chloroform containing 0, 1, 2, and 3% methanol (v/v, 200 mL each). The 1% methanol in the chloroform fraction was purified further with a Sep-Pak C18 cartridge column eluted with 70, 80, 90, and 100% methanol (5 mL each). The 100% methanol fraction was then subjected to reversed-phase HPLC (Senshu-Pak Pegasil-B, 4.6 × 250 mm) eluted with aqueous methanol as a mobile phase (0 - 10 min: 50%, 10 - 20 min: gradient to 100%, 20 - 70 min: 100% methanol) at a flow rate of 2.5 mL/min. Fractions were collected every minute. The HPLC fractions corresponding to authentic 6-oxoCHN (18 - 19 min) and cholest-4-en-3-one (37 - 39 min) were collected.

Enzyme preparation and assay. Young tomato plants (5 g) 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 13,000g. The resultant precipitate was then dissolved in assay buffer containing 0.1 M sodium phosphate (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 20% (v/v) glycerol, and used as the cell-free enzyme solution.

The reactions were initiated *via* the addition of substrates (CHR, cholest-4-en-3-one and CHN), followed by 30 min of incubation at 37 °C. The metabolites of the enzyme reactions were extracted with ethyl acetate (1.2 mL) and concentrated in vacuo. The ethyl acetate-soluble fraction was loaded onto a Sep-Pak C18 cartridge column, and washed sequentially 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 then subjected to a reversed-phase HPLC, as mentioned above. Finally, the enzyme products were analyzed *via* capillary GC-MS/SIM after suitable derivatization.

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

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