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RESEARCH PAPER

Biosynthesis of a cholesterol-derived brassinosteroid, 28-norcastasterone, in *Arabidopsis thaliana*

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Abstract

A metabolic study revealed that 28-norcastasterone in *Arabidopsis* is synthesized from cholesterol via the late C-6 oxidation pathway. On the other hand, the early C-6 oxidation pathway was found to be interrupted because cholestanol is converted to 6-oxocholestanol, but further metabolism to 28-norcathasterone was not observed. The 6-oxoBRs were found to have been produced from the respective 6-deoxoBRs administered to the enzyme solution, thus indicating that these 6-oxoBRs are supplied from the late C-6 oxidation pathway. Heterologously expressed CYP85A1 and CYP85A2 in yeast catalysed this C-6 oxidation, with CYP85A2 being much more efficient than CYP85A1. Abnormal growth of *det2* and *dwf4* was restored via the application of 28-norcastasterone and closer precursors. Furthermore, *det2* and *dwf4* could not convert cholesterol to cholestanol and cholestanol to 6-deoxo-28-norcathasterone, respectively. It is, therefore, most likely that the same enzyme system is operant in the synthesis of both 28-norcastasterone and castasterone. In the presence of *S*-adenosyl-L-methionine, the cell-free enzyme extract catalysed the C-24 methylation of 28-norcastasterone to castasterone, although the conversion rates of 28-norteasterone to teasterone and 28-nortyphasterol to typhasterol were much lower; this suggests that 28-norcastasterone is the primary precursor for the generation of C_{28}-BRs from C_{27}-BRs.

Key words: *Arabidopsis thaliana*, brassinosteroids, C_{27}-BRs biosynthesis, 28-norcastasterone.

Introduction

The absence of brassinosteroids (BRs) in the *Arabidopsis* mutants *det2*, *cpd*, and *dwf4* (Li *et al.*, 1996; Szekeres *et al.*, 1996; Choe *et al.*, 1998; Noguchi *et al.*, 1999), tomato *dwarf* (Bishop *et al.*, 1999), and pea *lkb* (Nomura *et al.*, 1997, 1999) results in pleiotropic abnormalities, including reduced shoot elongation, reduced fertility, delayed senescence, and altered vasculature and photomorphogenesis. Mutants can be restored to the wild-type phenotype via the application of BRs. Similar abnormalities are also observed in the *Arabidopsis* mutants *bri1* (Li and Chory, 1997), *bin2* (Li *et al.*, 2001; Li and Nam, 2002), and *bak1* (Nam and Li, 2002), as well as in the tomato mutant *curl-3* (Koka *et al.*, 2000). However, the mutant phenotype cannot be rescued by the application of BRs because of disrupted BR signalling. Therefore, BRs are currently regarded as essential plant hormones whose endogenous levels must be properly maintained in plant cells to facilitate normal growth and development.

Naturally-occurring BRs, the number of which totals over 50, can be classified into C_{27}-, C_{28}-, or C_{29}-BRs based on the nature of the alkyl groups occupying the C-24 position in the side chain of the 5α-cholestane carbon skeleton. Among them, the C_{28}-BRs that harbour a C-24 methyl group are major BRs in the plant kingdom. Castasterone (CS) and brassinolide (BL) belonging to the C_{28}-BRs are biologically highly active and, therefore, have been extensively investigated for their biosyntheses by means of feeding experiments as well as molecular genetics of BR-deficient mutants. According to the results, two parallel pathways—namely the early and late C-6-oxidation pathway in plant cells—have been proposed (Fujioka *et al.*, 1997; Yokota, 1997; Sakurai, 1999; Bishop and Yokota, 2001; Fujioka and Yokota, 2003; Fig. 1). The biosynthesis of C_{28}-BRs begins with the hydrogenation of campesterol to campestanol. In the early C-6 oxidation pathway, campestanol is then oxidized to 6-oxocampestanol, which undergoes successive oxidation to cathasterone (CT), teasterone (TE), 3-dehydroteasterone (3-DHT), typhasterol (TY), and CS. In the late C-6 oxidation pathway, campestanol is first oxidized at C-22 to generate 6-deoxocathasterone (6-deoxoCT), which is then oxidized successively to 6-deoxoteasterone (6-deoxoTE), 6-deoxo-3-dehydroteasterone (6-deoxo-3-DHT), 6-deoxotyphasterol (6-deoxoTY), 6-deoxocastasterone (6-deoxoCS), and CS. Finally, CS is oxidized to BL with a 7-oxalactone moiety.

28-Norcastasterone (28-norCS), a C_{27} counterpart of CS, has also been identified from as many as 12 plant tissues, although less frequently than CS (Fujioka, 1999; Fujioka *et al.*, 2000; Bajguz and Tretyn, 2003). 28-NorCS possesses the same carbon skeleton as cholesterol, thus suggesting that 28-norCS is synthesized from cholesterol in a fashion similar to the synthesis of CS from campesterol. Tomato seedlings were determined to contain cholesterol, cholestanol, and several 6-deoxo-28-norBRs including 6-deoxo-28-norcathasterone (6-deoxo-28-norCT), 6-deoxo-28-nortyphasterol (6-deoxo-28-norTY), and 6-deoxo-28-norcastasterone (6-deoxo-28-norCS) (Yokota *et al.*, 2001; Kim *et al.*, 2004*b*). In addition, the cell-free enzyme extract of tomato seedlings catalysed the conversion of cholesterol to cholestanol and 6-deoxo-28-norTE to 28-norCS via 6-deoxo-28-nor-3-DHT, 6-deoxo-28-norTY, and 6-deoxo-28-norCS. These findings demonstrate that the synthesis of 28-norCS is mediated by late C-6 oxidation (Kim *et al.*, 2004*b*). Furthermore, the cell-free enzyme extract mediated the C-24 methylation of 28-norCS to CS in the presence of NADPH and *S*-adenosyl-L-methionine (SAM). It was also determined that exogenously applied 28-norCS restores the abnormal growth of the tomato *dwarf* mutant which is defective in a cytochrome

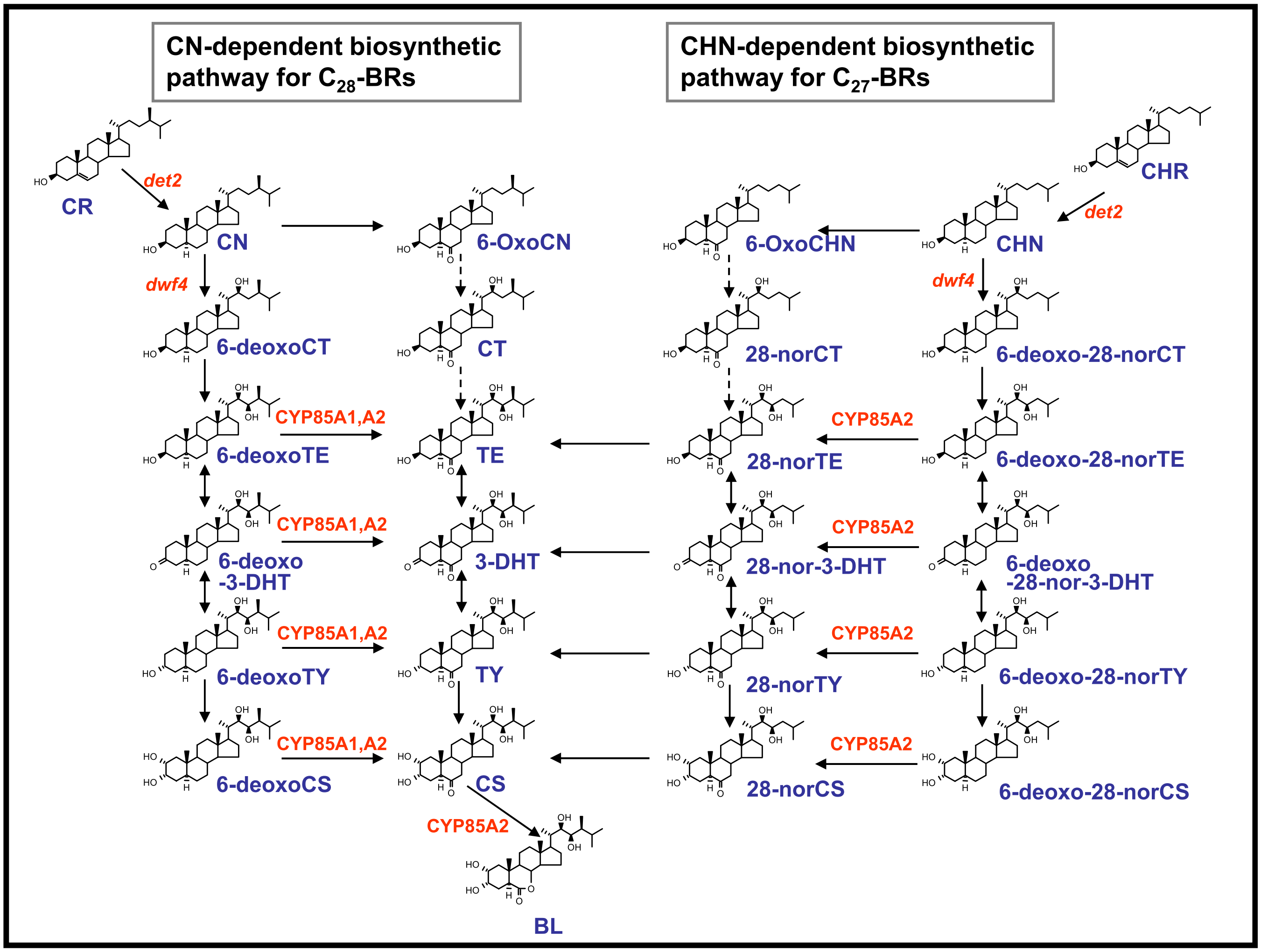

Fig. 1. Biosynthetic pathways for C_{27}- and C_{28}-BRs and their connection established in *A. thaliana*. The solid and dashed arrows indicate verified and not verified biosynthetic steps, respectively. The names on arrows indicate genes or enzymes catalysing biosynthetic reactions.

P450, CYP85A, involved in the C-6 oxidation of 6-deoxoCS and 6-deoxo-28-norCS to CS and 28-norCS, respectively. Therefore, 28-norCS is biologically important per se and is also important in the production of CS.

In *Arabidopsis*, C_{27}-BRs including 28-norTY and 28-norCS have been identified, in addition to the C_{28}-BRs (Fujioka *et al.*, 2000). The conversion of cholestanol to 6-oxocholestanol, as a possible upstream step in C_{27}-BRs biosynthesis, has also been demonstrated (Lee *et al.*, 2010), although downstream steps for the generation of C_{27}-BRs in *A. thaliana* have yet to be clearly elucidated.

Despite our previous efforts, the biosynthesis of C_{27}-BRs via the early C-6 oxidation pathway remains to be clearly characterized. Furthermore, the linkage of the early and late C-6 oxidation pathways of C_{27}-BRs, as well as the biosynthetic relationship between C_{27}- and C_{28}-BRs, is still not completely understood. In this study, these subjects were investigated using *Arabidopsis* enzyme extracts. The enzymes and genes involved in C_{27}-BRs biosynthesis have also been addressed.

Materials and methods

Plant growth conditions

Cold-treated seeds of wild-type *Arabidopsis* (Col-0) were planted in soil and grown for 3 weeks in an environmental growth chamber at 22 °C, under a 16 h light (120 μmol photons m^{-2} s^{-1})/20 °C, 8 h dark cycle. When seeds were planted on 1× MS medium (Duchefa, Haarlem, Netherlands) containing 0.8% (w/v) agar and 1% (w/v) sucrose, the seeds were surface-sterilized with 70% ethanol and a 30% (v/v) bleach solution containing 0.025% (v/v) Triton X-100.

Enzyme assays

3-week-old soil-grown *Arabidopsis* plants (20 g) were harvested and ground with a mortar and pestle in cold 0.1 M sodium phosphate (pH 7.4) buffer containing 15 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, 40 mM ascorbate, 250 mM sucrose, and 10% (v/v) glycerol. The homogenate was then centrifuged for 15 min at 8000 *g* to remove cell debris. The supernatant was then centrifuged for an additional 30 min at 20 000 *g*. The resultant supernatant was 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 centrifuged for an additional 10 min at 13 000 *g*. The resultant precipitate was 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. For microsomal preparation, the supernatant obtained from centrifugation at 20 000 *g* was subjected to 1 h of ultra-centrifugation at 100 000 *g*. The resultant pellet was re-suspended with assay buffer.

The enzyme assay mixture was composed of 5 μg of substrate, 3–-5 mg of enzyme solution, and the appropriate co-factor (NADP/NADPH) or co-substrate (*S*-adenosyl-L-methionine). The reactions were initiated via the addition of substrate and the incubation was conducted for 30 min at 37 °C. The metabolites of the enzyme reactions were extracted with ethyl acetate (1.2 ml, three times) and concentrated *in vacuo*. The ethyl acetate-soluble fraction was loaded onto a Sep-Pak C_{18} cartridge column (Waters, Milford, MA), and sequentially washed with 50% and 60% methanol (5 ml each). The fraction eluted with 100% methanol was concentrated *in vacuo*, dissolved in 50 μl of methanol, and then subjected to reversed phase (RP)-HPLC (Senshu Pak C_{18}, 10×150 mm) eluted at a flow rate of 2.5 ml min^{-1} with 100% methanol for the metabolites of cholesterol and cholestanol or acetonitrile (MeCN)–water gradients (0–20 min, 45% MeCN; 20–40 min, 45–100% MeCN; 40–70 min, 100% MeCN) for 6-deoxo-28-norBRs, or a flow rate of 2 ml min^{-1} with 60% MeCN for 28-norBRs. The fractions were collected every minute. The fractions (cholestanol, 18–19 min; 6-oxo-cholestanol, 7–8 min; 6-deoxo-28-norCT, 58–61 min; 6-deoxo-28-norTE, 44–46 min; 6-deoxo-28-nor-3-DHT, 46–48 min; 6-deoxo-28-norTY, 49–51 min; 6-deoxo-28-norCS, 36–38 min, 28-norTE, 27–29 min; 28-nor-3-DHT, 34–36 min; 28-norTY, 33–35 min; 28-norCS, 13–15 min) in which authentic BRs were detected under the same RP-HPLC conditions were analysed via GC-MS or GC-SIM after appropriate derivatization.

C-6 oxidations of C_{27}-and C_{28}-BRs by CYP85A1 and CYP85A2

CYP85A1/V60/WAT21 and CYP85A2/V60/WAT21 yeast strains were employed as previously described (Kim *et al.*, 2005*b*). 6-Deoxo-28-norBR and its counterpart, 6-deoxo-BR, (5 μg each) were fed to galactose-induced yeast cells and incubated for 6 h. [26,28-$^2H_6$]BR was added to the cell culture as an internal standard prior to extraction with ethyl acetate. Purification using a Sep-Pak C_{18} cartridge column was conducted in accordance with the method described above. The fraction eluted with 100% methanol was subjected to RP-HPLC (Senshu Pak C_{18}, 10×150 mm) eluted at a flow rate of 2.5 ml min^{-1} with MeCN–water gradients (0–20 min, 45% MeCN; 20–40 min, 45–100% MeCN; 40–60 min, 100% MeCN). The fractions (28-norTE, 27–29 min; 28-nor-3-DHT, 34–36 min; 28-norTY, 33–35 min; 28-norCS, 13–15 min; TE, 31–34 min; 3-DHT, 37–39 min; TY, 37–39 min; CS, 19–21 min) containing C_{27}-BR and C_{28}-BR were eluted and combined, and then subjected to GC-MS analysis. The quantities of the C_{28}-BRs metabolites were initially calculated using [26,28-$^2H_6$]BRs as an internal standard and the amounts of C_{27}-BRs, the counterparts of C_{28}-BRs, were estimated by the area ratio relative to C_{28}-BRs on the total ion chromatogram.

Sterol analysis

3-week-old soil-grown *Arabidopsis* plants (2 g fresh weight) were harvested and extracted with methanol:chloroform (4:1, v/v). The extracts were concentrated *in vacuo* and solvent-partitioned between chloroform and water. D_7 cholesterol (0.5 μg) was added to the chloroform-soluble fraction as an internal standard. The fraction extracted with *n*-hexane after alkaline hydrolysis was purified on a Sep-Pak silica cartridge column (Waters, Milford, MA) and subjected to GC-MS analysis.

GC-MS/SIM analysis

The GC-MS or GC-SIM analyses were conducted as previously described (Kim *et al.*, 2005*b*). The samples were subjected to methaneboronation or trimethylsilyation according to the structures of the expected metabolites. Methaneboronation was conducted by heating the samples dissolved in pyridine containing methaneboronic acid (2 mg ml^{-1}) at 80 °C for 30 min and *N*-methyl-*N*-TMS-trifluoroacetamide (MSTFA, Pierce, Rockford, IL) was used for trimethylsilylation.

Rescue experiments of det2 and dwf4 mutants by C_{27}-BRs

The *det2, dwf4*, and wild-type (Col-0 or En-2) seeds were surface-sterilized and planted on 1× MS agar plates containing 1 μM of various C_{27}-BR biosynthetic intermediates or mock solution. After 5 d under continuous darkness, the seedlings (*n* >30) were photographed with a digital camera and the lengths of the hypocotyls were measured with Scion Image software (Scion Corporation, Maryland, USA).

Results

Arabidopsis enzymes were extracted with phosphate buffer containing the appropriate additives prior to centrifugation, and successive precipitation with acetone. The precipitates were then dissolved in assay buffers and employed as crude enzyme extracts for *in vitro* conversion experiments. Unlabelled substrates were used for enzymatic incubation, since isotope-labelled substrates were not available. The absence of the expected products in the prepared enzyme extracts was confirmed via GC-MS and GC-SIM prior to incubation with the substrates. The enzyme products were purified via RP-HPLC and then derivatized to trimethylsilyl ethers (TMSi), bismethaneboronates (BMB) or methaneboronate-trimethylsilyl ethers (MB-TMSi). These derivatives were rigorously characterized by GC-MS and/or GC-SIM analyses.

Biosynthesis 6-deoxo C_{27}-BRs in A. thaliana

The late C-6 oxidation pathway for 28-norCS proceeds through the following sequence: cholesterol → cholestanol → 6-deoxo-28-norCT → 6-deoxo-28-norTE → 6-deoxo-28-nor-3-DHT → 6-deoxo-28-norTY → 6-deoxo-28-norCS → 28-nor-CS. The presence of this pathway has been demonstrated, although not fully clarified, in the tomato. By way of contrast, no evidence has yet been obtained supporting the existence of such a pathway in *A. thaliana*.

Our *Arabidopsis* enzyme extracts catalysed the conversion of cholesterol to cholestanol, which is consistent with our findings that cholesterol and cholestanol are endogenous in *Arabidopsis* plants (Table 1). However, the incubation of cholestanol and 6-deoxo-28-norCT in the crude *Arabidopsis* enzyme extract did not result in any of the expected metabolites. However, enzymes prepared from microsomes catalysed the conversion of cholestanol to 6-deoxo-28-norCT and 6-deoxo-28-norTE (Table 2). The enzymes that convert cholestanol to 6-deoxo-28-norCT and 6-deoxo-28-norTE do not appear to be abundant in *A. thaliana*. On the other hand, the crude enzyme extract catalysed the conversion of 6-deoxo-28-norTE to 6-deoxo-28-nor-3-DHT and 6-deoxo-28-norTY, of 6-deoxo-28-nor-3-DHT to 6-deoxo-28-norTE and 6-deoxo-28-norTY, and of 6-deoxo-28-norTY to 6-deoxo-28-nor-3-DHT and 6-deoxo-28-norTE; these results indicate that the epimerization of C-3 from 6-deoxo-28-norTE to 6-deoxo-28-norTY occurs via 6-deoxo-28-nor-3-DHT, in a reversible fashion. The metabolites of 6-deoxo-28-norTY also included 6-deoxo-28-norCS, demonstrating that the enzyme extract harbours C2 α-hydroxylase. Finally, 6-deoxo-28-norCS was metabolized to 28-norCS by the same enzyme extract. It has also been established that the late C-6 oxidation pathway, which produces 28-norCS, is operant in *A. thaliana,* as anticipated.

Biosynthesis of 6-oxo C_{27}-BRs in A. thaliana

Table 1. Content of major 4-demethylasterols in *A. thaliana*

	Amount (μg g^{-1} fresh weight) 1st experiment	2nd experiment
Cholesterol	6.60	7.94
Cholestanol	0.38	1.45
Campesterol	22.41	21.06
Campestanol	1.21	1.02
Stigmasterol	3.62	4.84
Sitosterol	107.13	99.45
Sitostanol	8.35	9.68

If the early C-6-oxidation pathway of C_{27}-BRs exists in *A. thaliana*, 28-norCS will be synthesized according to the following sequence: cholestanol → 6-oxocholestanol → 28-norCT → 28-norTE → 28-nor-3-DHT → 28-norTY → 28-nor-CS. It was determined that our *Arabidopsis* enzyme extracts catalysed the conversion of cholestanol to 6-oxocholestanol (Table 2). However, 28-norCT was not detected in the cholestanol metabolites, and thus this metabolism was investigated further using enzymes prepared from microsomes obtained via ultra-centrifugation. Nonetheless, 28-norCT, as well as further metabolites including 28-norTE, were not produced in the reaction mixture as shown by the results of GC-SIM analysis. It appears most likely that the pathway from 6-oxocampestanol to 28-norCT is blocked in *Arabidopsis*.

The feeding of 28-norTE to the enzyme extract resulted in the production of 28-nor-3-DHT and 28-norTY, whereas the feeding of 28-norTY gave rise to 28-nor-3-DHT and 28-norTE, thereby indicating that 28-norTE and 28-norTY are interconvertible via 28-nor-3-DHT (Table 2). Furthermore, 28-norCS was identified as another metabolite of 28-norTY (Table 2). Therefore, the pathway connecting 28-norTE to 28-noCS was determined to be present in *A. thaliana*.

Biosynthetic connection of 6-deoxo and 6-oxo C_{27}-BRs in A. thaliana

Two parallel pathways—the early and late C-6 oxidation pathways of C_{28}-BRs—are biosynthetically connected by the C-6 oxidation of 6-deoxoTE, 6-deoxo-3-DHT, and 6-deoxoTY to TE, 3-DHT, and TY, respectively. In *Arabidopsis*, AtBR6ox1 (CYP85A1) and AtBR6ox2 (CYP85A2) mediate these C-6 oxidations (Kim *et al.*, 2005*b*). An attempt was made to determine whether CYP85A1 and CYP85A2 are involved in any possible biosynthetic connection between the early and late C-6 oxidation pathways of C_{27}-BRs. To this end, the cDNA of *Arabidopsis* CYP85A1 and CYP85A2 were cloned into a galactose-inducible expression vector, pYeDP60 (V60), and transformed into the WAT21 yeast strain, wherein the expression of *Arabidopsis* NADPH-Cyt P450 reductase is inducible by galactose (Pompon *et al.*, 1996; Urban *et al.*, 1997). After confirming that the C-6 oxidation of 6-deoxo-28-norCS did not occur in the empty vector-transformed yeast (V60/WAT21), 6-oxidations of C_{27}- and C_{28}-BRs were evaluated by the transformed strains (CYP85A1/V60/WAT21 and CYP85A2/V60/WAT21).

Both CYP85A1/V60/WAT21 and CYP85A2/V60/WAT21 successfully catalysed the 6-oxidation of C_{27}-BRs,

Table 2. GC-MS data of metabolites obtained from *A. thaliana* cell-free conversion experiments

Substrate	Metabolite	RRt[a]	Prominent Ions
CHR	CHN[b]	0.456	460(M+, 54), 445(73), 370(28), 355(43), 305(33), 215(100)
CHN	6-oxoCHN[c]	0.495	474 (M+, 19), 459(51), 445(100), 384(4), 159(8)
	6-deoxo-28-norCT	0.542	533(M+-15, 1), 368(2), 255(8), 173(100)
	6-deoxo-28-norTE	0.605	516(M+, 69), 501(55), 459(23), 426(26), 411(39), 305(35), 230(27), 215(100), 141(30)
28-norTE	28-nor-3-DHT	0.876	456(M+, 90), 399(3), 316(19), 286(13), 245(35), 141(100)
	28-norTY	0.735	530(M+, 60), 515(35), 501(100), 440(56), 425(21), 229(16), 141(21)
	TE	1.031	544(M+, 29), 529(53), 515(100), 454(5), 300(8), 155(39)
28-nor-3-DHT	3-DHT	1.013	470(M+, 63), 399(7), 357(5), 316(21), 298(10), 287(11), 245(11), 155(100)
28-norTY	28-nor-3-DHT	0.876	456(M+, 90), 399(2), 316(16), 286(11), 245(36), 141(100)
	28-norTE	0.906	530(M+, 21), 515(53), 501(100), 440(3), 316(16), 141(11)
	28-norCS	0.866	498(M+, 100), 483(8), 399(4), 358(12), 328(7), 287(36), 141(52)
	TY	0.863	544(M+, 100), 529(81), 515(55), 454(72), 300(10), 155(60)
28-norCS	CS	1.000	512(M+, 80), 358(33), 327(12), 287(32), 155(100)
6-deoxo-28-norTE	6-deoxo-28-nor-3-DHT	0.615	442(M+, 73), 427(10), 246(12), 231(100), 217(23), 163(20), 141(15)
	6-deoxo-28-norTY	0.523	516(M+, 23), 501(6), 459(4), 426(62), 411(60), 305(11), 230(30), 215(100), 141(24)
	28-norTE	0.906	530(M+, 21), 515(50), 501(100), 440(5), 316(18), 141(12)
6-deoxo-28-nor-3-DHT	6-deoxo-28-norTE	0.605	516(M+, 73), 501(65), 459(25), 426(23), 411(36), 305(38), 230(26), 215(100), 141(17)
	6-deoxo-28-norTY	0.523	516(M+, 21), 501(5), 459(4), 426(60), 411(59), 305(10), 230(32), 215(100), 141(24)
	28-nor-3-DHT	0.876	456(M+, 91), 399(3), 316(20), 286(13), 245(33), 141(100)
6-deoxo-28-norTY	6-deoxo-28-nor-3-DHT	0.615	442(M+, 74), 427(10), 246(12), 231(100), 217(22), 163(20), 141(14)
	6-deoxo-28-norTE	0.605	516(M+, 71), 501(62), 459(25), 426(23), 411(35), 305(36), 230(26), 215(100), 141(23)
	28-norTY	0.735	530(M+, 60), 515(34), 501(100), 440(55), 425(24), 229(15), 141(23)
	6-deoxo-28-norCS	0.619	484(M+, 51), 469(16), 288(15), 273(100), 205(24), 141(21)
6-deoxo-28-norCS	28-norCS	0.866	498(M+, 100), 483(3), 399(4), 358(12), 328(7), 287(36), 141(54)

[a] RRt: relative retention time on GC.
[b] The sample was analysed as BMB.
[c] The sample was analysed as BMB-TMSi ether.

6-deoxo-28-norTE, 6-deoxo-28-nor-3-DHT, 6-deoxo-28-norTY, and 6-deoxo-28-norCS to 28-norTE, 28-nor-3-DHT, 28-norTY, and 28-norCS, respectively (Fig. 2A). The respective conversion rates were 17, 14, 68, and 24 times higher in CYP85A2/V60/WAT21 than in CYP85A1/V60/WAT21.

Similarly, when C_{28}-BRs, 6-deoxoTE, 6-deoxo-3-DHT, 6-deoxoTY, and 6-deoxoCS were fed to the transformed yeast strains, TE, 3-DHT, TY, and CS were detected as the respective products (Fig. 2B). The rates of conversion by CYP85A2/V60/WAT21 were also higher than the rates of conversion by CYP85A1/V60/WAT21, although to lesser extents than were noted in the 6-oxidations of C_{27}-BRs. No 6-oxidations occurred in the feedings of the biosynthetically upstream intermediates, campestanol, cholestanol, 6-deoxoCT, and 6-deoxo-28-norCT.

An attempt was also made to determine whether *Arabidopsis* enzyme extracts are capable of converting 6-deoxo C_{27}-BRs to 6-oxo C_{27}-BRs. As shown in Table 2, 6-deoxo-28-norTE, 6-deoxo-28-nor-3-DHT, 6-deoxo-28-norTY, and 6-deoxo-28-norCS were 6-oxidized to 28-norTE, 28-nor-3-DHT, 28-norTY, and 28-norCS, respectively. However, the 6-oxidation of 6-deoxo-28-norCT to 28-norCT was not detected in the enzyme extracts. These findings are consistent with those obtained using the transformed yeast strains.

Demethylation of 28-norCS

An attempt was made first to characterize the *Arabidopsis* enzymatic activity that converts 28-norCS to 28-norBL. However, we were unable to find any such an activity in the enzyme extract. Rather, it was determined that 28-norCS was converted to a compound with a molecular ion of *m*/*z* 484 as a BMB derivative. The molecular ion was 14 mass units smaller than that of the 28-norCS BMB derivative, which suggests the loss of a methyl group (Table 2). A prominent ion at *m*/*z* 127, which is derived from the side chain due to the fission of the C20–C22 bond, is also 14 mass units smaller than the corresponding ion of 28-norCS BMB. The presence of ions at *m*/*z* 358, 328, and 287 shows the ring structure to be identical to that of 28-norCS. It is, therefore, likely that one of the methyls was lost in the side chain. The loss of C-26 has been reported in previous metabolic studies of BRs (Kim *et al.*, 2000, 2004*a*). Thus, the most probable structure of this metabolite is 26,28-dinorCS (Fig. 3).

Conversion of C_{27}-BRs to C_{28}-BRs through C-24 methylation

C_{27}-BRs were incubated with the *Arabidopsis* enzyme extracts in the presence of *S*-adenosyl-L-methionine and NADPH, and their conversion to C_{28}-BRs was assessed. The administration of 28-norCS yielded CS, as shown by a full-scan mass spectrum (Table 2). The administration of 28-norTE, 28-nor-3-DHT, and 28-norTY generated TE, 3-DHT, and TY, respectively, as identified by GC-SIM. Their conversion rates ranged from 0.2–0.3%, and were approximately 20–30-fold lower than that of the C-24 methylation of 28-norCS to CS (Table 3).

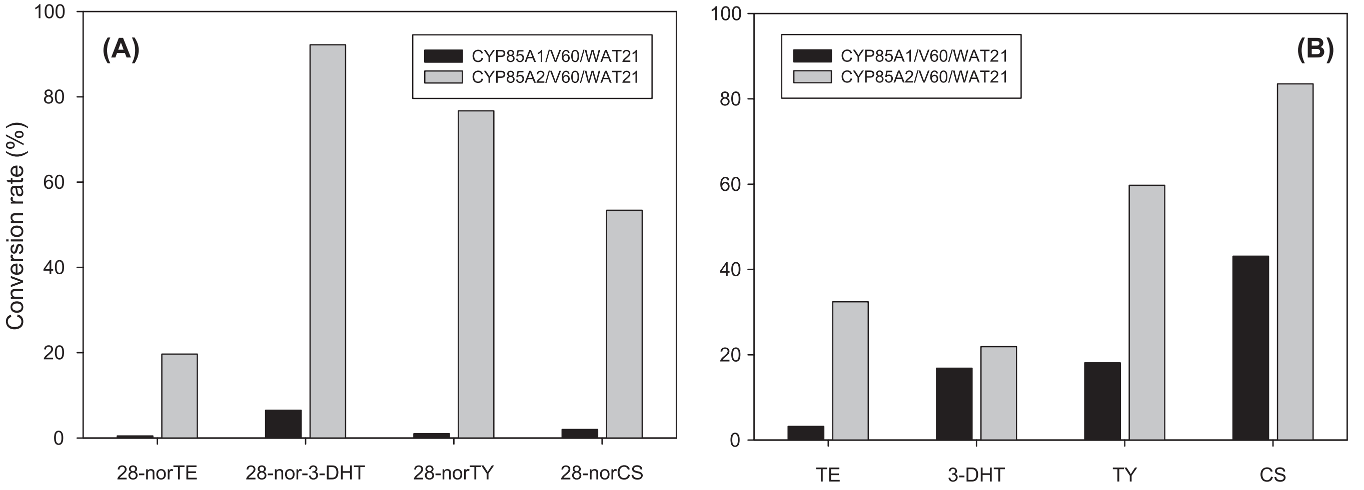

Fig. 2. Comparison of BR C-6 oxidase activity in CYP85A1/V60/WAT21 and CYP85A2/V60/WAT21 strains. (A) C-6 oxidation for C_{27}-BRs, (B) C-6 oxidation for C_{28}-BRs.

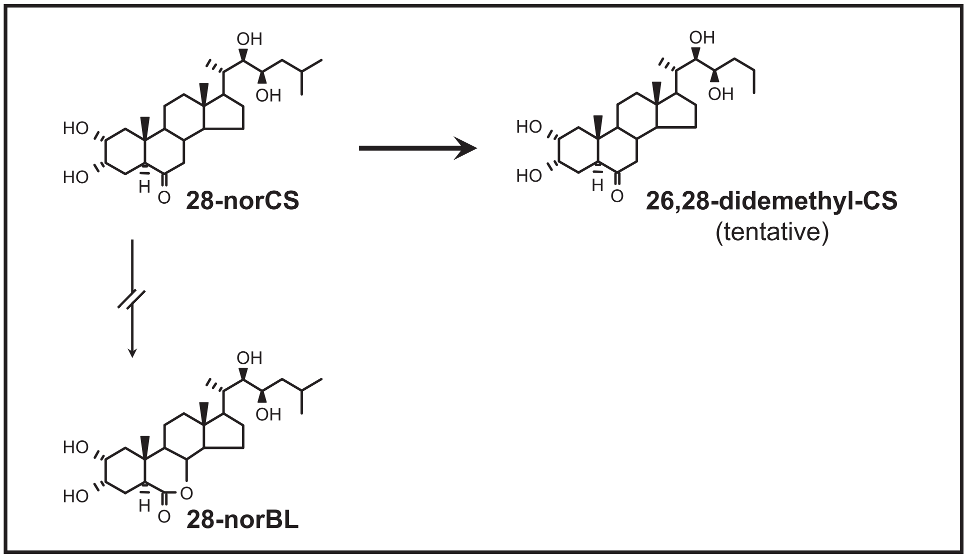

Fig. 3. Metabolism of 28-norCS in *Arabidopsis*. 28-NorCS converted to 26,28-didemethyl-CS (tentative), but not to 28-norBL.

Growth recovery by and biosyntheses of C_{28}-BRs in the Arabidopsis *mutants* det2 *and* dwf4

The restoration of growth in the BR-deficient mutants, *det2* and *dwf4*, was evaluated in dark-grown seedlings via the application of C_{27}-sterols and C_{27}-BRs.

The *det2* mutant was rescued by biosynthetically downstream C_{27}-BRs in the early and late C-6 oxidation pathway, such as 6-deoxo-28-norCT, 6-deoxo-28-norTE, 6-deoxo-28-norTY, 28-norTE, 28-norTY, and 28-norCS, with more downstream BRs being more biologically active (Fig. 4A). A similar growth recovery rate was also observed in the *dwf4* mutant (Fig. 4B).

Table 3. GC-MS/SIM data for C24-methylation of 28-norTE, 28-nor-3-DHT, 28-norTY, 28-norCS to TE, 3-DHT, TY, and CS in the presence of SAM and NADPH

Substrate	Metabolite	Conversion rate (%)
28-norTE	TE	0.2
28-nor-3-DHT	3-DHT	0.2
28-norTY	TY	0.3
28-norCS	CS	6.0

In an effort to investigate the role of the *DET2* gene encoding for steroid 5α-reductase in C_{27}-BRs biosynthesis, enzyme extracts were prepared from the wild-type Col-0 and the mutant *det2,* and were fed on cholesterol. The conversion of cholesterol to cholestanol was detected in Col-0 (Fig. 5A) but not in *det2* (Fig. 5B), thereby indicating that the *DET2* gene is involved in the conversion of cholesterol to cholestanol, and hence in the biosynthesis of C_{27}-BRs.

The *DWF4* gene encoding for steroid 22-hydroxylase was also evaluated for the conversion of cholestanol to 6-deoxo-28-norCT using the enzyme extract prepared from

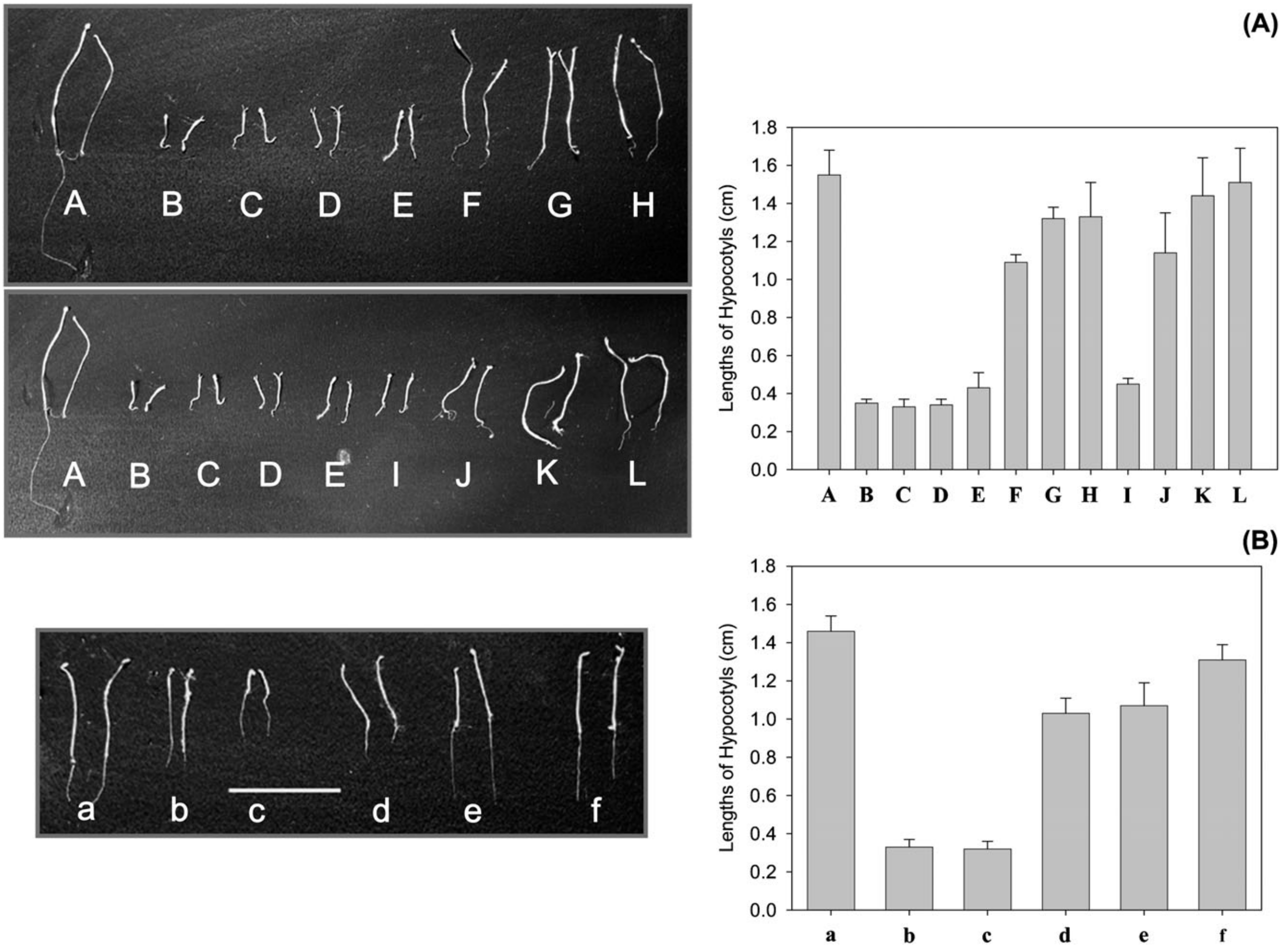

Fig. 4. Growth recovery of *det2* (A) and *dwf4* (B) by C_{27}-sterols and C_{27}-BRs. (A) A, Col-0/B-L, *det2*; B, Control; C, *det2*+cholesterol; D, *det2*+Cholest-4-en-3-one; E, *det2*+cholestanol; F, *det2*+6-deoxo-28-norCT; G, *det2*+6-deoxo-28-norTE; H, *det2*+6-deoxo-28-norTY; I, *det2*+6-oxocholestanol; J, *det2*+28-norTE; K, *det2*+28-norTY; L, *det2*+28-norCS. (B) a, Wild-type (En-2); b, *dwf4*; c, *dwf4*+6-oxocholestanol; d, *dwf4*+28-norTE; e, *dwf4*+28-norTY; f, *dwf4*+28-norCS. Error bars donate standard errors (*n* >30).

the wild-type En-2 and the mutant *dwf4*. The enzyme extract from En-2 successfully catalysed the conversion of cholestanol to 6-deoxo-28-norCT (Fig. 5C), but that from *dwf4* did not (Fig. 5D), thereby indicating that the *DWF4* gene is involved in the biosynthesis of C_{27}-BRs.

Discussion

It was reported previously that the endogenous level of 28-norCS (0.24 ng g^{-1} fresh weight) in *Arabidopsis* reaches a level approximately one-eighth that of CS (2.01 ng g^{-1} fresh weight) (Kim *et al.*, 2005*b*). Furthermore, it has been demonstrated that a change as small as 20% in the endogenous level of CS can induce phenotypic alternations, thereby suggesting that C_{27}-BRs including 28-norCS must play an important role in the growth and development of *Arabidopsis* (Kim *et al.*, 2005*b*; Kwon *et al.*, 2005). In seedlings of *Arabidopsis*, cholesterol, the parent sterol of 28-norCS, is contained at one-third the levels of campesterol, the parent sterol of CS (Table 1); this indicates that *Arabidopsis* contains a sufficient reservoir of cholesterol for use in the synthesis of 28-norCS.

Biosynthetic pathway of C_{27}-BRs via late C-6 oxidation

In this study, it has been demonstrated, using *Arabidopsis* seedlings, that the synthesis of 28-norCS from cholesterol occurs via the late C-6 oxidation pathway: cholesterol → cholestanol → 6-deoxo-28-norCT → 6-deoxo-28-norTE ↔ 6-deoxo-28-nor-3-DHT ↔ 6-deoxo-28-norTY → 6-deoxo-28-norCS → 28-norCS. The same biosynthetic pathway has been tentatively proposed in the tomato plant (Yokota *et al.*, 2001).

Recent biochemical studies conducted by Ohnishi *et al.* (2006, 2009) have demonstrated that the CYP90B1-mediated 22-hydroxylation of campesterol is an important first step in the synthesis of C_{28}-BRs in *Arabidopsis*. Campesterol and cholesterol were found to be favourable substrates for this enzyme, when compared with campestanol and cholestanol (Fujita *et al.*, 2006). It is therefore assumed that the formation of 22-hydroxycholesterol from cholesterol is an important step in 28-norCS

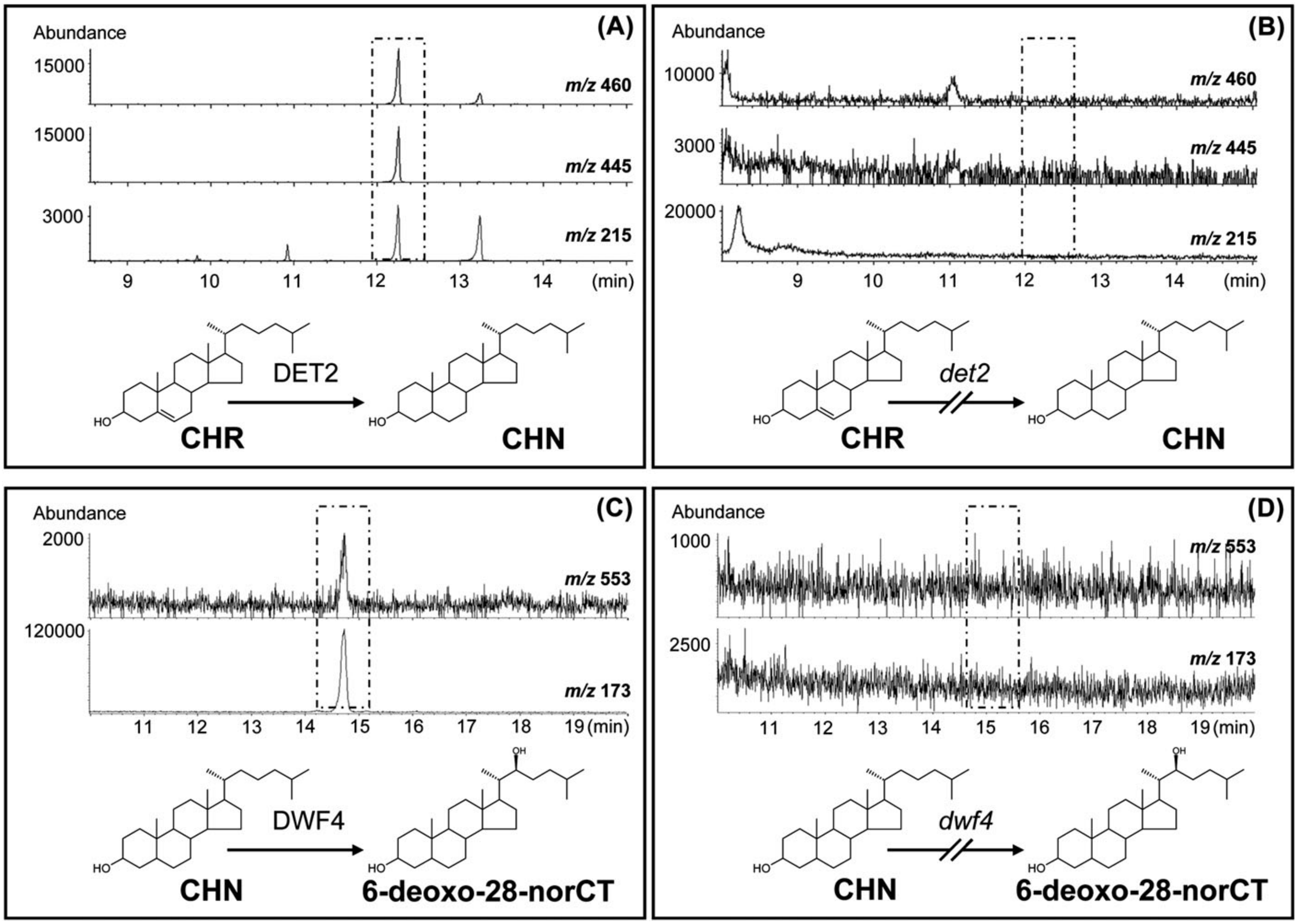

Fig. 5. GC-SIM analysis conversion of cholesterol to cholestanol and cholestanol to 6-deoxo-28-norCT in *det2* (B) and *dwf4* (D), respectively. (A) Conversion of cholesterol to cholestanol in Col-0, the wild type of *det2*. (C) Conversion of cholestanol to 6-deoxo-28-norCT in En-2, the wild type of *dwf4*.

biosynthesis. The biosynthesis of C_{27}-BRs, starting with the 22-hydroxylation of cholesterol, is currently being investigated.

The early C-6 oxidation pathway of C_{27}-BRs is blocked and 6-oxoBRs is derived from respective 6-deoxoBRs

It was determined that the early C-6 oxidation pathway halted at the stage of 6-oxocholestanol because its presumed metabolite, 28-norCT, was not generated after incubation with a microsomal enzyme preparation. In addition, endogenous 28-norCT we could not be identified, even using as much as 30 kg fresh weight of *Arabidopsis* plants (data not shown). However, *Arabidopsis* contained enzymes that converted 28-norTE to 28-nor-3-DHT, 28-norTY, and 28-norCS successively, thereby indicating that the BRs belonging to the early C-6 oxidation pathway are supplied by respective 6-deoxoBRs. Among the enzymes responsible for C-6 oxidation of C_{27}-BRs, CYP85A2 was determined to be 15 times as active as CYP85A1 in the C-6 oxidation of C_{27}-BRs, thereby indicating that CYP85A2 performs a central function in the C-6 oxidation of C_{27}-BRs (Fig. 2A). CYP85A2 has been determined to be more powerful than CYP85A1 in the C-6 oxidation of C_{28}-BRs (Kim *et al.*, 2005b). CYP85A2 also exhibits BL synthase activity (Kim *et al.*, 2005*b*; Kwon *et al.*, 2005; Nomura *et al.*, 2005). However, CYP85A2 did not catalyse the 7-oxalactonation of 28-norCS to 28-norBL, thereby suggesting that CYP85A2 is specific for the conversion of CS to BL.

Disproof against the early C-6 oxidation pathway of C_{28}-BRs

Some evidence has accumulated against the notion that the early C-6 oxidation pathway plays a role in C_{28}-BR biosyntheses. The first step of this pathway is the 6-oxidation of campestanol to 6-oxocampestanol, which has previously been identified from *Catharanthus* crown gall cells (Fujioka and Sakurai, 1997). However, since that time, the occurrence of 6-oxocampestanol in other plants has yet to be confirmed. It has been determined that CYP85A1 and CYP85A2, which are known as BR 6-oxidases, did not catalyse this reaction, leaving the responsible enzyme to be determined (Shimada *et al.*, 2001; Kim *et al.*, 2005b; Kwon *et al.*, 2005). Furthermore, the 22-hydroxylation of 6-oxocampestanol to CT has yet to be confirmed even in

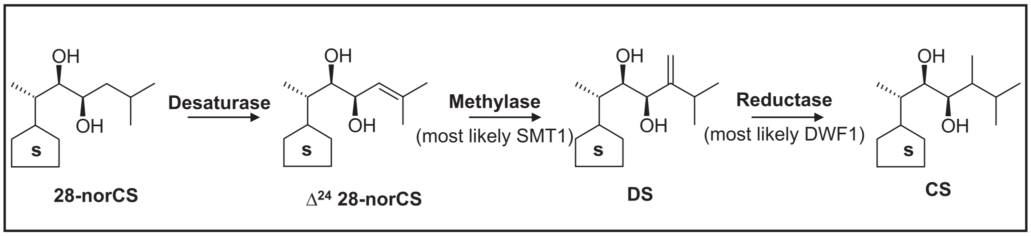

Fig. 6. A proposed scheme for the three step C-24 methylation of 28-norCS to CS in the presence of SAM and NADPH in *Arabidopsis*. S indicates the same ring structure as that of 28-norCS and CS.

Catharanthus crown gall cells, although CT was endogenous in the cells (Fujioka *et al.*, 1995). The conversion of 6-oxocampestanol to CT, as well as the presence of CT in any other plants, has yet to be demonstrated (Fujioka *et al.*, 1995; Joo *et al.*, 2002). Recently, Fujita *et al.* (2006) demonstrated that DWF4 (CYP90B1) 22-hydroxylated campestanol, but not 6-oxocampestanol. Altogether, our results indicate that the early C-6 oxidation pathway is commonly interrupted in plant tissues.

Biosyntheses of C_{27}- and C_{28}-BRs are catalysed by the same enzymes

Biosynthetic reactions occurring in C_{27}-BRs biosynthesis, including 5α-reduction, C-22 hydroxylation, C-23 hydroxylation, C-3 epimerization, C-2 α-hydroxylation, and C-6 oxidation, are exactly the same as those occurring in C_{28}-BRs biosynthesis. This may suggest that the same enzymes mediate the same reactions in the biosyntheses of both C_{28}-BRs and C_{27}-BRs. In support of this notion, heterologously-expressed CYP85A1 and CYP85A2 involved in the C-6 oxidation of C_{28}-BRs exert the same activity in the biosynthesis of C_{27}-BRs. The *det2* mutant cannot 5α-hydrogenate campesterol, and also cannot 5α-hydrogenate cholesterol (Fig. 5B), whereas the *dwf4* mutant catalyses the 22*R*-hydroxylation of neither campestanol nor cholestanol. Moreover, the abnormal growth of *det2* and *dwf4* mutants was successfully restored via the exogenous application of downstream C_{27}-BRs. Collectively, the findings of this study suggest that C_{27}-BRs and C_{28}-BRs biosynthesis are most likely controlled by the same biosynthetic enzymes.

CS synthesis from 28-norCS via methylation

It has been determined that *Arabidopsis* enzyme extract can methylate 28-norCS to CS. This constitutes a supplement to our earlier report demonstrating the presence of the same enzymatic activity in the tomato (Kim *et al.*, 2004*b*). It appears that this methylation reaction may be a ubiquitous event in the plant kingdom. As shown in Fig. 6, using the tomato plant, it was determined that this reaction occurs via the following three steps: (i) desaturation of 28-norCS to form Δ^{24}-28-norCS, (ii) SAM-dependent methylation of Δ^{24}-28-norCS to form dolichosterone (DS), and (iii) NADPH-dependent reduction of DS by NADPH to form CS (Kim *et al.*, 2004*b*). The SAM-dependent methylation is presumed to be catalysed by sterol methyltransferase 1 (SMT1). The NADPH-dependent reduction will be controlled by the *DWF1* gene in *Arabidopsis* or its orthologue gene, *OsDWF2,* in rice. In support of this, the *Osdwf2* rice mutant accumulates DS (Hong *et al.*, 2005). Additional evidence was recently obtained, using a *P. vulgaris* enzyme extract, that NADPH is required for the conversion of DS to CS (Joo *et al.*, 2009). It was found that 28-norCS is far more readily methylated than 28-norTE and 28-norTY in *Arabidopsis*, which indicates that C_{27}-BRs and C_{28}-BRs are connected largely through the passage from 28-norCS to CS (Fig. 1). In order to confirm the presence of these steps in *Arabidopsis*, metabolic and molecular genetic studies using relevant mutants are currently underway.

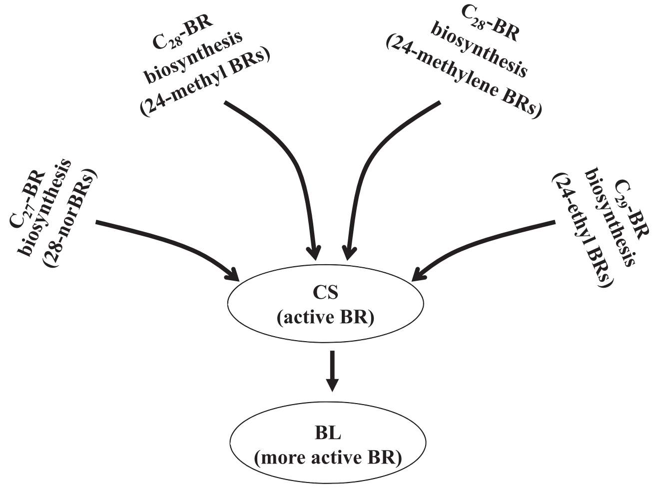

Fig. 7. Biosynthetic connection of C_{27}-BRs (28-norBRs), C_{28}-BRs (24-methylene BRs and 24-methyl BRs) and C_{29}-BRs (24-ethyl BRs) in plants. The multiple BRs biosynthetic pathways are funneled into CS to show BR activity in plant growth and development.

Deactivation of 28-norCS through demethylation

28-NorCS fed to the *Arabidopsis* enzyme extract was not only methylated, but also demethylated. It has been demonstrated previously in several plants that CS and BL are deactivated via C-26 demethylation into 26-norCS and 26-norBL (Kim *et al.*, 2000; 2004*a*). Therefore, the demethylation product of 28-norCS is tentatively designated as 26,28-norCS. Such demethylation events appear to perform a crucial role in regulating the levels of 28-norCS, which is regarded as biologically active per se (Kim *et al.*, 2005*a*).

In conclusion, it has been demonstrated here that multiple biosynthetic pathways lead to CS. Recently, Joo *et al.* (2009) determined that, in *P. vulgaris*, DS is hydrogenated to CS. Our recent study (unpublished) revealed another biosynthetic pathway from 28-homoCS to CS via C-28 demethylation. Thus, it is most conceivable that all the biosynthetic pathways of BRs in plants are funnelled into CS to carry out the relevant biological activities (Fig. 7).

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