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 C28-BRs from C27-BRs.

Key words: Arabidopsis thaliana, brassinosteroids, C27-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 bkk1 (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 C27-, C28-, or C29-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 C28-BRs that harbour a C-24 methyl group are major BRs in the plant kingdom. Castasterone (CS) and brassinolide (BL) belonging to the C28-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., 2004b). 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., 2004b). 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.](image-url)
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*, C27-BRs including 28-norTY and 28-norCS have been identified, in addition to the C28-BRs (Fujioka et al., 2000). The conversion of cholesterol to 6-oxocholesterol, as a possible upstream step in C27-BRs biosynthesis, has also been demonstrated (Lee et al., 2010), although downstream steps for the generation of C27-BRs in *A. thaliana* have yet to be clearly elucidated.

Despite our previous efforts, the biosynthesis of C27-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 C27-BRs, as well as the biosynthetic relationship between C27- and C28-BRs, is still not completely understood. In this study, these subjects were investigated using *Arabidopsis* enzyme extracts. The enzymes and genes involved in C27-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 30% (v/v) bleach solution containing 0.025% (v/v) Triton X-100. The resultant pellet was re-suspended with assay buffer. The supernatant obtained from centrifugation at 20000 × g, 30 min at 4 °C, was subjected to GC-MS analysis. The fractions were collected every minute. The fractions (cholesterol, 18–19 min; 6-oxo-cholesterol, 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.

#### 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 resultant precipitate was dissolved in methanol, and a 30% (v/v) bleach solution containing 0.25% (v/v) Triton X-100 was added to the cell culture as an internal standard prior to extraction with ethyl acetate. Purification using a Sep-Pak C18 cartridge column was conducted in accordance with the method described above. The fraction eluted with 100% methanol was subjected to RP-HPLC (Senshu Pak C18, 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 C27-BR and C28-BR were eluted and combined, and then subjected to GC-MS analysis. The quantities of the C28-BRs metabolites were initially calculated using [26,28-2H₆]BRs as an internal standard and the amounts of C27-BRs, the counterparts of C28-BRs, were estimated by the area ratio relative to C28-BRs on the total ion chromatogram.
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 C27-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-norCS. The presence of this pathway has been demonstrated, although not fully clarified, in the tomato. By way of contrast, Cs. The presence of this pathway has been demonstrated, although not fully clarified, in the tomato.

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-oxocholestanol, 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-norCS was determined to be present in A. thaliana.

Biosynthetic connection of 6-deoxo and 6-oxo C27-BRs in A. thaliana

Two parallel pathways—the early and late C-6 oxidation pathways of C27-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., 2005b). 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 C27-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 C27- and C28-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 C27-BRs.

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

| Substance    | 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          |
| 28-norTE     | 1.36                        | 1.07          |
| 28-norTY     | 1.02                        | 0.94          |
| 28-norCS     | 1.56                        | 1.45          |

Notes: The results of GC-SIM analysis. It appears most likely that the pathway from 6-oxocampestanol to 28-norCS 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-norCS was determined to be present in A. thaliana.

Biosynthesis of 6-oxo C27-BRs in A. thaliana

If the early C-6-oxidation pathway of C27-BRs exists in A. thaliana, 28-norCS will be synthesized according to the following sequence: cholesterol → 6-oxocholestanol → 28-norCT → 28-norTE → 28-nor-3-DHT → 28-norTY → 28-norCS. It was determined that our Arabidopsis enzyme extracts catalysed the conversion of cholesterol 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-norCS 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-norCS was determined to be present in A. thaliana.

Two parallel pathways—the early and late C-6 oxidation pathways of C27-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., 2005b). 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 C27-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 C27- and C28-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 C27-BRs,
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 derivative, which 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, 2004a). Thus, the most probable structure of this metabolite is 26,28-dinorCS (*Fig. 3*).

### Conversion of C27-BRs to C28-BRs through C-24 methylation

C27-BRs were incubated with the *Arabidopsis* enzyme extracts in the presence of S-adenosyl-L-methionine and NADPH, and their conversion to C28-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*).
Growth recovery by and biosyntheses of C28-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 C27-sterols and C27-BRs.

The det2 mutant was rescued by biosynthetically downstream C27-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).

In an effort to investigate the role of the DET2 gene encoding for steroid 5α-reductase in C27-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 C27-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

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                 |

Fig. 2. Comparison of BR C-6 oxidase activity in CYP85A1/V60/WAT21 and CYP85A2/V60/WAT21 strains. (A) C-6 oxidation for C27-BRs, (B) C-6 oxidation for C28-BRs.

Fig. 3. Metabolism of 28-norCS in Arabidopsis. 28-NorCS converted to 26,28-didemethyl-CS (tentative), but not to 28-norBL.
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 C27-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., 2005b). 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 C27-BRs including 28-norCS must play an important role in the growth and development of Arabidopsis (Kim et al., 2005b; Kwon et al., 2005). In seedlings of Arabidopsis, cholesterol, the parent sterol of 28-norCS, is contained at one-third the levels of campsterol, 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 C27-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 campsterol is an important first step in the synthesis of C28-BRs in Arabidopsis. Campsterol and cholesterol were found to be favourable substrates for this enzyme, when compared with campstanol 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
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_{28}-BRs (Kim et al., 2005b). CYP85A2 also exhibits BL synthase activity (Kim et al., 2005b; 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 (Fujikochi 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...
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 C27- and C28-BRs are catalysed by the same enzymes**

Biosynthetic reactions occurring in C27-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 C28-BRs biosynthesis. This may suggest that the same enzymes mediate the same reactions in the biosyntheses of both C28-BRs and C27-BRs. In support of this notion, heterologously-expressed CYP85A1 and CYP85A2 involved in the C-6 oxidation of C28-BRs exert the same activity in the biosynthesis of C27-BRs. The det2 mutant cannot 5α-hydrogenate campesterol, and also cannot 5α-hydrogenate cholesterol (Fig. 5B), whereas the dwf4 mutant catalyses the 22R-hydroxylation of neither campestanol nor cholesterol. Moreover, the abnormal growth of det2 and dwf4 mutants was successfully restored via the exogenous application of downstream C27-BRs. Collectively, the findings of this study suggest that C27-BRs and C28-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., 2004b). 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 Δ24,28-norCS, (ii) SAM-dependent methylation of Δ24,28-norCS to form dolichosterone (DS), and (iii) NADPH-dependent reduction of DS by NADPH to form CS (Kim et al., 2004b). 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 C27-BRs and C28-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.

**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; 2004a). 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., 2005a).
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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