Journal of Plant Biology, December 2005, 48(4) : 483-486

REPORT

Endogenous Level of 28-Norcastasterone Is Strictly Regulated in Plant Cells

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A cell-free enzyme solution prepared from cultured cells of *Phaseolus vulgaris* mediated C-24 methylation of 28-norcastasterone to castasterone with the aid of S-adenosylmethionine as a co-substrate in the presence of the NADPH cofactor. This enzyme solution also catalyzed conversion of 28-norcastasterone to a demethylated 28-norcastasterone, most likely 26,28-didemethyl-castasterone, when S-adenosylmethionine was not added to the enzyme solution. Furthermore, gene expression of *Arabidopsis CYP85A1* and *CYP85A2* mediating the conversion of 6-deoxo-28-norcastasterone to 28-norcastasterone was strongly inhibited by treatment of 28-norcastasterone. These results suggest that 28-norcastasterone, along with castasterone and brassinolide, is an important brassinosteroid whose endogenous level should be strictly controlled to express brassinosteroid activities in plants.

Keywords: Arabidopsis, brassinosteroid, homeostatic regulation, 28-norcastasterone, *Phaseolus vulgaris*

Brassinosteroids (BRs) are steroidal plant hormones that regulate growth and development of plants (Yokota, 1997; Bajguz and Tretyn, 2003). They are classified as C_{27}-, C_{28}-, and C_{29}-BRs based on their number of carbon atoms (Yokota, 1997). Among C_{27}-BRs, 28-norcastasterone (28-norCS) has been most frequently identified in the plant kingdom (Fujioka, 1999; Bajguz and Tretyn, 2003). Recently, we used a cell-free enzyme solution obtained from young tomato plants to demonstrate that 28-norCS is biosynthesized from a C_{27}-phytosterol, cholesterol via 6-deoxo type C_{27}-BRs (Kim et al., 2004a). Additionally, this solution successfully mediated conversion of 28-norCS to CS, an active C_{28}-BR in young tomato plants. This suggests that 28-norCS is a C_{27}-BR that is biologically important for maintaining an endogenous level of active BRs, such as CS or/and brassinolide (BL), in plants. Nevertheless, the mechanisms involved in the control of endogenous levels of 28-norCS in plants are not yet well known. This has prompted us to investigate the metabolism of 28-norCS in cultured cells of *Phaseolus vulgaris* where 28-norCS has been identified (data will be published separately). We are also examining the effect of 28-norCS on the expression of genes that encode enzymes involved in BR biosynthesis.

Here, cultured cells (7 g) of *P. vulgaris* were homogenized with Na-phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 15 mM 2-mercaptoethanol, 15% glycerol, 250 mM sucrose, 1% polyvinylpyrrolidone, and 40 mM ascorbate. They were centrifuged at 8,000g for 10 min. The supernatant was then re-centrifuged at 20,000g for 30 min. Cold acetone (final concentration 40%) was added to the resulting supernatant. After incubation at −25°C for 10 min, the solution was centrifuged at 13,000g for 10 min. The obtained precipitate was re-dissolved in 0.1 M Na-phosphate buffer containing 1.5 mM 2-mercaptoethanol and 30% glycerol, and used as a crude enzyme solution for assay of 28-norCS metabolism.

Because no detectable amount of BRs is contained in the crude enzyme solution (Kim et al., 2001), non-isotope labeled 28-norCS and *S*-adenosylmethionine (SAM) were added as substrates to the solution in the presence of NADPH to examine whether 28-norCS can be converted into CS in the *Phaseolus* cells. After incubation at 37°C for 30 min, the assay was terminated by the addition of ethyl acetate (1.2 mL). The ethyl acetate soluble fraction was then concentrated to dryness, dissolved in 50% methanol, and loaded on a C_{18} Sep-Pak cartridge eluted with 50% and 100% methanol (5 mL each). The 100% methanol fraction was dried, dissolved in a small amount of

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Table 1. GC-MS data for authentic CS and a metabolite of 28-norCS in cultured cells of *P. vulgaris*.

Compound*	Rt** (min) on GC	Prominent ions (*m/z*, relative intensity %)
Metabolite	28.26	512 (M+, 72), 441 (11), 358 (36), 327 (12), 287 (31), 155 (100)
Authentic CS	28.26	512 (M+, 75), 441 (11), 358 (38), 327 (13), 287 (34), 155 (100)

*Sample was analyzed as its bismethaneboronate derivative.
**Retention time.

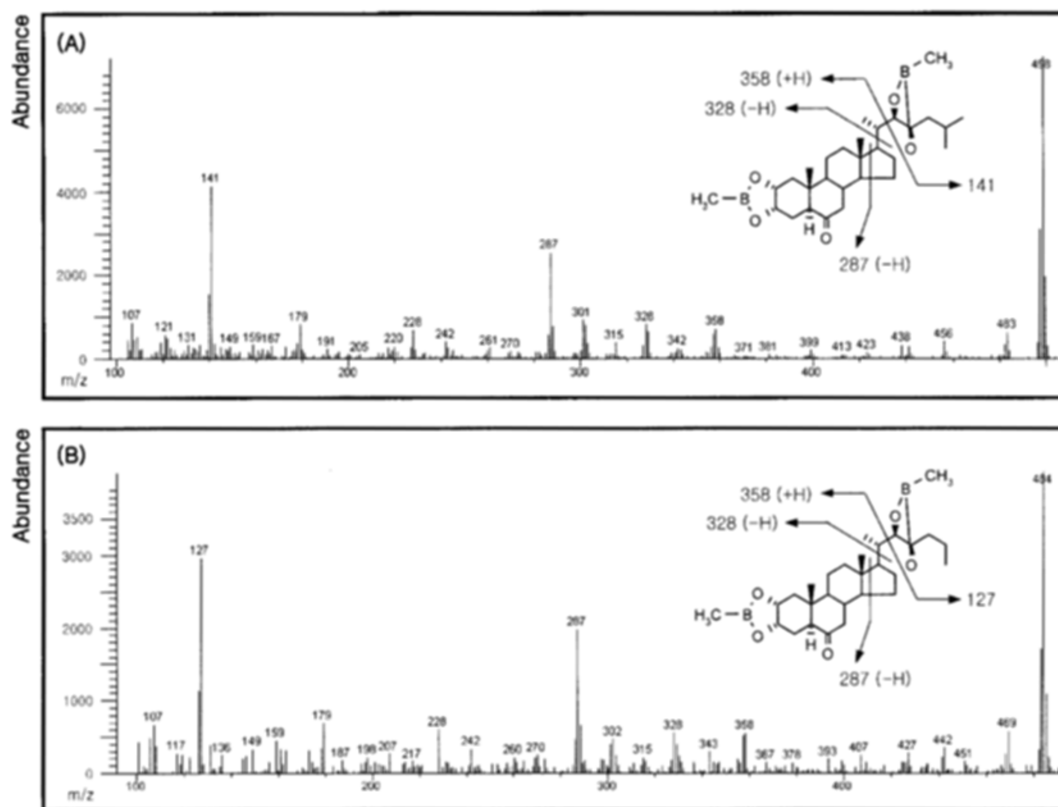

Figure 1. Mass spectrum and fragmentation pattern of BMB of 28-norCS **(A)** and its catabolite **(B)**.

methanol, and subjected to reversed phase HPLC (Nova Pak C_{18}, 8 X 100 mm) at a flow rate of 1 mL min^{-1}, eluted with 45% acetonitrile. Fractions collected every minute were analyzed by capillary GC-MS (Kim et al., 2001). HPLC Fractions 13 and 14, which corresponded to the retention time of CS under the same HPLC condition were dried and treated with methaneboronic acid in pyridine (1 mg mL^{-1}). The obtained bismethaneboronate (BMB) of the active compound in the fractions showed the same GC retention time and mass spectrum as those of authentic CS BMB, indicating that the active compound is CS (Table 1). Therefore, the conversion of 28-norCS to CS was verified in the cell-free enzyme solution.

It has been demonstrated with cell-free enzyme extracts that two C_{28}-BRs, BL and CS, are catabolized into 26-norBL and 26-norCS, respectively, in plants (Kim et al., 2000, 2004b). Therefore, we used the aforementioned enzyme mixture, without the addition of SAM, to investigate the possibility for C-26 demethylation of 28-norCS in *Phaseolus* cells. After the assay, the product was purified by reversed phase HPLC (Pegasil-B ODS 10 X 100 mm) at a flow rate of 2.5 mL min^{-1}, eluted with 45% acetonitrile. Fractions collected every minute were analyzed by capillary GC-MS (Kim et al., 2001). In GC-MS, the BMB of a compound in HPLC fractions 9 through 11 gave a molecular ion at *m/z* 484 ($C_{28}H_{46}O_5B_2$). Providing that the compound was a demethylated 28-norCS, this is indeed 14 (CH_2) mass-reduced compared with that of the BMB of 28-norCS used as a substrate (Fig. 1). The BMB of the compound showed ions at *m/z* 358 ($C_{22}H_{35}O_3B$), 328 ($C_{20}H_{29}O_3B$), and 287 ($C_{17}H_{24}O_3B$),

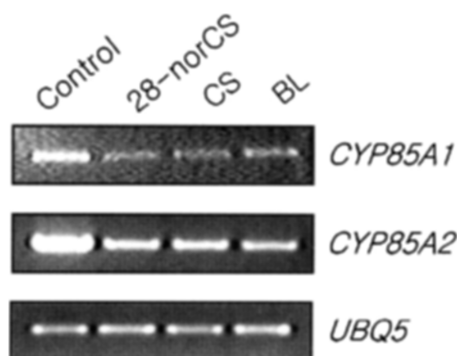

Figure 2. RT-PCR analysis of *CYP85A1* (At5g38970) and *CYP85A2* (At3g30180) gene expression in BRs-treated *Arabidopsis*. *UBQ5* (ubiquitin 5, At3g62250) expression indicates equal amounts of cDNA for each lane.

which indicated that the ring structure of the compound is identical to that of 28-norCS. This also demonstrated that a demethylation of 28-norCS does not occur in the ring structure, but in the side chain. Therefore, we believe that the compound is either 21, 28-di-demethyl-CS or 26, 28-di-demethyl-CS. However, a prominent ion due to fission of C20-C22 at *m/z* 127 ($C_6H_{12}O_2B$), which is also 14 (CH_2) mass-reduced compared with that derived from 28-norCS BMB, showed that the demethylation occurs at the propyl group on the end of the side chain, C-26. Taken together, we have tentatively characterized this compound as 26,28-di-demethyl-CS.

CS and BL inhibit the expression of *Arabidopsis* genes, e.g., *DWF4, CPD, CYP85A1*, and *CYP85A2*, which encode enzymes that catalyze earlier steps in C_{28}-BRs biosynthesis (Mathur et al., 1998; Shimada et al., 2003). To ascertain whether the feedback inhibition of C_{27}-BRs also occurs by 28-norCS, we investigated the effect of 28-norCS on the expression of *Arabidopsis CYP85A1* and *CYP85A2* mediating conversion of 6-deoxo-28-norCS to 28-norCS in the biosynthesis of C_{27}-BRs. RT-PCR was carried out using RNA extracts from BR-treated *Arabidopsis* seedlings, and the products were subsequently identified by DNA gel-blot analysis. Expression of both genes was strongly inhibited by treatment of 28-norCS (Fig. 2), indicating that higher-than-necessary levels of 28-norCS in plants induce feedback regulatory inhibition of an earlier biosynthetic step to maintain a steady-state level.

Our findings provide evidence that 28-norCS is catabolized into a demethylated 28-norCS, most likely 26,28-didemethyl-CS in *P. vulgaris*. Coupled with our previous findings that CS and BL are biodegraded into 26-norCS and 26-norBL, respectively, in plants (Kim et al., 2000, 2004b), we propose that C-26 demethylation is a common catabolic process to maintain a homeostatic level of biologically active BRs in plants. Nevertheless, the natural occurrences of demethylated catabolites have not been yet described.

These results demonstrate that an endogenous level of 28-norCS, an end product of the biosynthesis of C_{27}-BRs, is effectively reduced by feedback regulation of biosynthetic enzymes, conversion to CS, and a

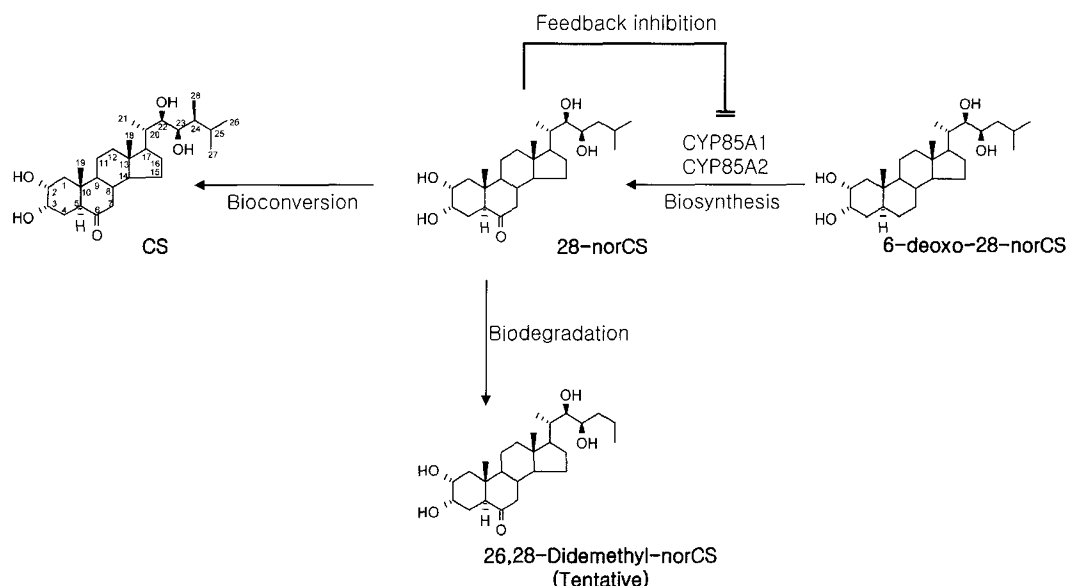

Figure 3. Proposed scheme for control of steady-state level of 28-norCS in plants.

catabolic process by C-26 demethylation (Fig. 3). Such regulatory features are the same as those shown in the homeostatic regulation of CS and BL, both of which are the most critical C_{28}-BRs in plants (Mathur et al., 1998; Kim et al., 2000, 2004a; Shimada et al., 2003). Therefore, along with CS and BL, 28-norCS should be considered an important BR whose endogenous level must be strictly regulated for the expression of BR activities in plants.

ACKNOWLEDGEMENT

This research was supported by a Korea Research Foundation Grant (KRF-2002-C00091).

Received June 15, 2005; accepted September 1, 2005.

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