Cell-free Conversion of Castasterone in Cultured Cells of *Phaseolus vulgaris* and *Marchantia polymorpha*

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Brassinosteroids (BRs) are steroidal plant hormones, which are required for the normal growth and development of plants. Castasterone (CS) and brassinolide (BL) are the most frequently identified BRs in the plant kingdom.[1,2] Feeding experiments using isotope-labeled substrates revealed that BL is biosynthesized from CS by 7-oxalactonation.[3,4] However, many plants which possess a fair amount of CS contain very low levels of BL.[1,2] In addition, in plants which can convert CS to BL, the conversion rate is extremely low, yielding high levels of CS in the plants.[1,5] Because CS, as well as BL, is known to induce feedback regulation of earlier steps in BR biosynthesis pathways,[6] accumulation of CS may limit or alter BR biosynthesis in plants. Therefore, endogenous levels of CS should be reduced, after BL production, to a level below that at which feedback regulation can occur. This is a difficult proposition, as little is yet known about the catabolism of CS. This dearth of available data prompted us to investigate the catabolism of CS in cultured *Phaseolus vulgaris* and *Marchantia polymorpha* cells, in which the presence of CS and BL and conversion of CS to BL have been demonstrated.[1,2,7]

Cultured cells (5 g) of *P. vulgaris* and *M. polymorpha* were homogenized[7] and centrifuged at 8,000 × g for 10 minutes, and the resulting supernatants were re-centrifuged at 20,000 × g for 30 min. Cold acetone was then added to the obtained supernatants (final volume 40%), and the acetone precipitates were re-suspended in 0.1 M Na phosphate buffer (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% glycerol for crude enzyme solutions.

Non-labeled CS and NADPH (4.8 mM) were added to the enzyme solutions as a substrate and a cofactor, respectively, to examine catabolism of CS in the plants. After incubating at 37 °C for 30 minutes, the assay mixtures were extracted with ethyl acetate (1.2 mL × 3). The obtained ethyl acetate soluble fractions were loaded on a Sep-Pak C_{18} cartridge eluted with 50%, and 100% methanol (5 mL each). The 100% methanol fractions were further purified by reversed phase HPLC (Nova Pak, C_{18}, 8 × 100 mm) and eluted with 40% acetonitrile at a flow rate of 1 mL min^{-1}. Fractions were collected every min, and analyzed by a preparative TLC (Merck, HPTLC F_{254}) developed with a 6 : 1 mixture of chloroform and methanol. Besides fraction 19-21, which contained CS (added as the substrate), fraction 13-15 exhibited a BR-like blue-purplish spot at R_f 0.30. The metabolite in the fractions was analyzed by GC-MS/-SIM after methaneboronation.

In GC-MS, bismethaneboronate (BMB) of the metabolite showed a molecular ion at *m/z* 498 and the most abundant ion at *m/z* 141, due to the fission of C20/C22, which was reduced in mass compared with CS BMB. The mass reduction suggests that a methyl in CS was eliminated in the metabolite. Therefore, the metabolite was proposed to be either 26-norCS or 28-norCS. In GC-MS, BMB of 26-norCS and 28-norCS showed basically the same mass spectrum, but their retention times on GC were clearly different. As shown in Table 1, GC retention time of BMB of the metabolite was equal to that of 26-norCS BMB. Consequently, the metabolite was characterized as 26-norCS. 26-NorCS showed approximately one-tenth the level of activity of CS, indicating that 26-norCS is a catabolite of CS in plant cells. Because isotope-labeled 26-norCS is not available, activity for the enzyme catalyzing the conversion of CS to 26-norCS, namely CS C-26 demethylase, was measured by GC-SIM based quantification method, yielding 0.90 and 0.31 ng mg^{-1}

Table 2. Activity of CS 6-oxidase and CS C-26 demethylase in cultured cells of *P. vulgaris* and *M. polymorpha*

Enzyme	Activity[a]	
	P. vulgaris	*M. polymorpha*
CS 6-oxidase	0.09	0.03
CS C-26 demethylase	0.90	0.31

[a]Enzyme activity was expressed as ng product mg $protein^{-1}min^{-1}$

Table 1. GC-MS/SIM data for authentic BRs and CS metabolite in cultured cells of *P. vulgaris* and *M. polymorpha*

Compound	R_t* on GC (min)	Prominent ions (*m/z*, relative intensity %)
CS Metabolite**	23.52	498(100), 483(9), 358(28), 328(11), 287(44), 141(93)
Authentic 26-norCS**	23.52	498(100), 483(9), 358(28), 328(11), 287(44), 141(93)
Authentic 28-norCS**	22.63	498(100), 483(8), 358(16), 328(11), 287(42), 141(52)

*R_t: Retention time. **The sample was analyzed by GC-MS as a bismethaneboronate

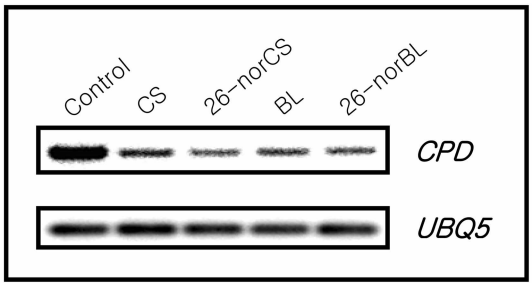

Figure 1. Feedback inhibition of Arabidopsis *CPD* expression by application of BRs. RT-PCR was performed using RNA extracted from BR-treated seedlings. Products were subsequently identified by DNA gel-blot analysis. *UBQ5* (ubiquitin 5) expression indicates equal amounts of cDNA for each lane.

min^{-1} in the *Phaseolus* and *Marchantia* cells, respectively (Table 2).

It has been demonstrated that CS is converted into a more active BR, namely BL, in cultured cells of *P. vulgaris* and *M. polymorpha*.[2,7] This study is the first to demonstrate that CS is simultaneously catabolized into a less biologically active 26-norCS in the plant cells. To determine which reaction predominantly occurs in the plant cells, activity of CS 6-oxidase, the enzyme mediating the conversions of CS to BL, was subsequently examined with the same enzyme solution. After finishing the assay, [26,28-$^2H_6$] BL was added for quantitative analysis, and the product was analyzed by GC-MS. Activity for CS 6-oxidase in the *Phaseolus* cells was determined to be 0.09 ng mg $protein^{-1}$ min^{-1}, and CS 6-oxidase activity in *Marchantia* cells was measured to be 0.03 ng mg $protein^{-1}$ min^{-1}.

It was known that CS and BL inhibit expression of *Arabidopsis thaliana* genes, such as *DWF4*, *CPD*, CYP85A1 and CYP85A2, which encode enzymes catalyzing earlier steps in BR biosynthesis.[9,10] To ascertain whether the C-26 demethylation of BR diminishes feedback regulation, the effects of 26-norCS and 26-norBL on expression of *Arabidopsis CPD* were examined. *Arabidopsis CPD* encodes a C-23(*R*) hydroxylase mediated conversion of cathasterone to teasterone, and 6-deoxocathasterone to 6-deoxoteasterone. As shown in Figure 1, application of 26-norCS and 26-norBL, as well as CS and BL, to *Arabidopsis* strongly inhibited the *CPD* gene expression, indicating that CS/BL feedback regulatory inhibition is not influenced by C-26 demethylation.

Our findings constitute the first evidence that CS is catabolized into 26-norCS in *Phaseolus* and *Marchantia* cells (Figure 2). Coupled with our previous finding that CS is converted into BL in both plants, this indicates that extra CS that is not converted into BL is catabolized into 26-norCS to maintain a homeostatic level of CS in the cells. The specific activity for CS demethylase was measured to be ten times higher than that of CS 6-oxidase in both plants, suggesting that higher levels of CS than plants need is more effectively reduced by C-26 demethylation than by conversion of CS to BL.

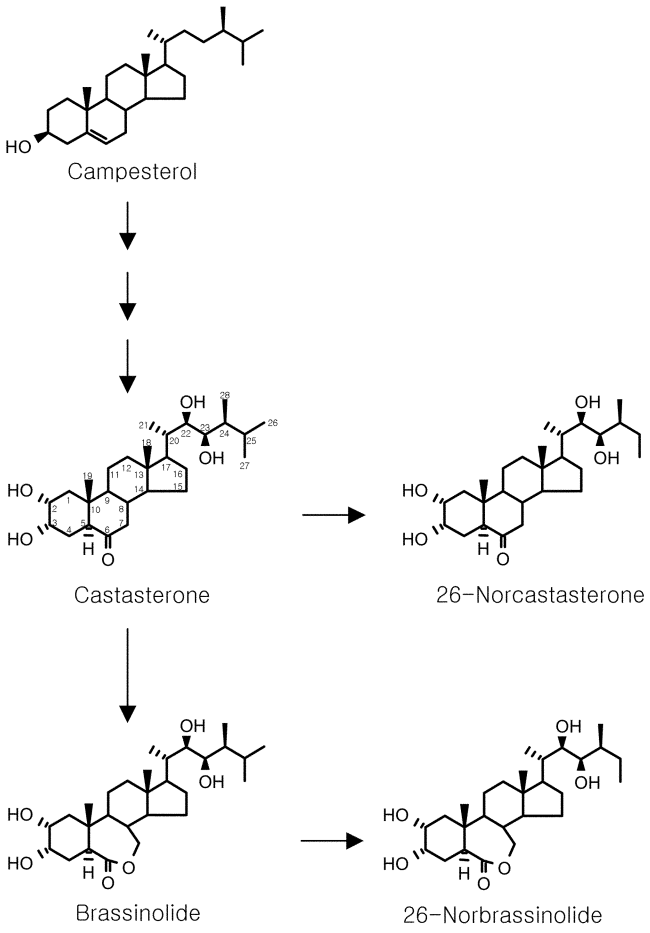

Figure 2. Biosynthesis and biodegradation of CS and BL in cultured cells of *P. vulgaris* and *M. polymorpha*.

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