

C-26 Demethylation of Brassinosteroids in *Arabidopsis thaliana*Seung-Hyun Son,[†] Ji Hyun Youn,[†] Min-Kyun Kim, and Seong-Ki Kim^{*}

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Steroidal plant hormones, collectively referred to as brassinosteroids (BRs), control a variety of phenomena in the growth and development of plants.¹⁻³ To exert hormonal activity, endogenous levels of BRs must be maintained in plants. Biosynthesis and biodegradation are regarded as primary factors that control endogenous BR levels. As the result of biochemical and molecular genetic studies, the biosynthetic pathways relevant to the generation of the physiologically active BRs, castasterone (CS) and brassinolide (BL), are now well-established.^{4,5} Additionally, many of the genes and proteins involved in these biosynthetic pathways have been identified.^{4,6} In contrast, the biodegradative processes that reduce BR activity have yet to be clearly identified.

Using enzyme solutions prepared from *Phaseolus vulgaris* and *Marchantia polymorpha*, we previously demonstrated that CS and BL are converted to 26-norCS and 26-norBL, respectively.^{7,8} 26-norBL has been shown to be less biologically active than BL, suggesting that C-26 demethylation of BRs is a degradation reaction that reduces the activity of biologically active BRs.^{7,8} Nevertheless, the natural occurrence of C-26 demethylated BRs, such as 26-norCS or 26-norBL, has yet to be demonstrated, which raises the question of whether or not C-26 demethylation actually functions in

the biodegradation of BRs. To obtain conclusive evidence regarding BR degradation, the existence of 26-demethylated BRs in *Arabidopsis thaliana* was assessed, resulting in the identification of 26-norCS. In this study, we report on the characterization and biosynthesis of 26-norCS in *A. thaliana*. Additionally, the C-26 demethylation of other BRs in the plant is described.

BRs in *Arabidopsis thaliana* (30 kg) was extracted and purified as described in Experimental Section. After a reverse-phase HPLC, fractions corresponding to synthetic 26-norBL (HPLC fraction I) and 26-norCS (HPLC fraction II) in the same HPLC were collected, derivatized to be bismethaneboronate (BMB), and analyzed by a capillary GC-MS/SIM.

HPLC fraction I showed no peak for 26-norBL-BMB on the total ion chromatogram. Further, no characteristic ions for 26-norBL-BMB were detected even by GC-SIM analysis (data not shown). This indicates that either there is no 26-norBL or that the endogenous level of 26-norBL is too low to be detected by GC-MS/SIM in *A. thaliana*.

When HPLC fraction II was analyzed by GC-MS, two peaks were detected at 20.11 and 18.95 min which yielded nearly identical mass spectra at m/z 498 (M^+), 483, 441, 399, 358, 327/328, 287, 141 (base ion peak) (Table 1). The mass ions for the molecular and base peaks were reduced by 14

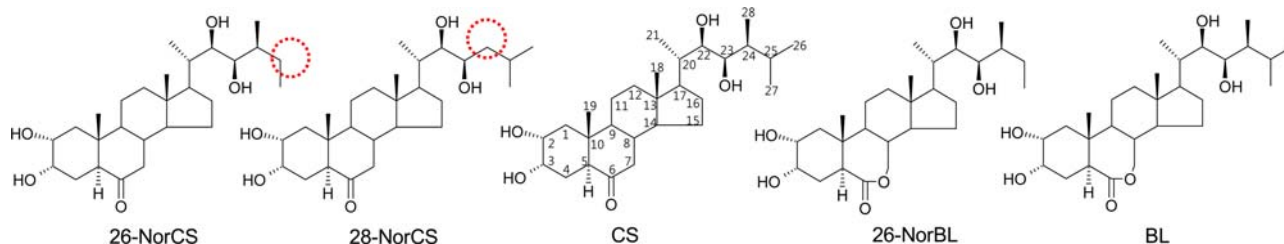


Figure 1. Chemical structure for 26-norCS, 28-norCS, CS, 26-norBL and BL described in this study. Numbers indicate the position of carbon.

Table 1. Identification of 26-norCS and 28-norCS in *A. thaliana* by a capillary GC-MS analysis

Compound ^a	Rt ^b on GC	MS prominent ion (relative intensity, %)
Arabidopsis 26-norCS	20.11	498 (M^+ , 100), 483(8), 441(3), 399(4), 358(12), 327/328(7), 287(36), 141(52)
Arabidopsis 28-norCS	18.95	498 (M^+ , 100), 483(6), 441(4), 399(7), 358(13), 327/328(8), 287(33), 141(54)
Synthetic 26-norCS	20.11	498 (M^+ , 100), 483(7), 441(3), 399(4), 358(12), 327/328(7), 287(36), 141(54)
Synthetic 28-norCS	18.95	498 (M^+ , 100), 483(8), 441(5), 399(8), 358(15), 327/328(5), 287(36), 141(56)

^aThe sample was analyzed as a bismethaneboronate. ^bRt: retention time (min)

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mass units relative to those derived from CS-BMB. The prominent ions at m/z 358, 327/328 and 287 are characteristic ions derived from the ring structure of CS-BMB. Therefore, the compounds are CS analogues demethylated at the side chain, most likely at C-26 and C-28. Next, synthetic 26-norCS and 28-norCS were analyzed *via* GC-MS under the same analysis conditions described above. As summarized in Table 1, the BMB of the demethylated CS seen at 20.11 min had an identical mass spectrum and GC retention time as that of 28-norCS-BMB, which identified the demethylated CS as 28-norCS. The BMB of the other demethylated CS seen at 18.95 min had an identical mass spectrum and GC retention time to 26-norCS, thus identifying the compound as 26-norCS. As a consequence, the presence of two C₂₇-BRs - 26-norCS and 28-norCS - was demonstrated in *A. thaliana*.

It has been previously reported that 26-norCS and 26-norBL have approximately one-tenth less biological activity relative to CS and BL, respectively.^{4,9} As it has been already demonstrated that CS and BL were converted to 26-norCS and 26-norBL, respectively, in *Phaseolus vulgaris* and *Marchantia polymorpha*,^{7,8} it was attempted to confirm by *in vitro* enzyme assay that such conversions take place in Arabidopsis. As shown in Figure 2(a) and 2(c), conversion of CS to 26-norCS and BL to 26-norBL was demonstrated by a capillary GC-MS analysis.

The conversions of 26-norCS to CS and 26-norBL to BL were also examined using the same enzyme assay in the presence of *S*-adenosylmethionine as a methyl donor. As shown in Figure 2(b) and 2(d), both conversions for 26-norCS to CS and 26-norBL to BL were not detected in Arabidopsis, providing that conversions of CS to 26-norCS and BL to 26-norBL are irreversible in *A. thaliana*.

In this study, we demonstrated the occurrence of 26-norCS in *A. thaliana*. This is the first study to show the presence of 26-nor-type BRs in plants. In the rice lamina inclination assay, 26-norCS showed lower levels of biological activity than CS.⁹ Additionally, the enzyme solution prepared from

A. thaliana successfully catalyzed the conversion of CS to 26-norCS. These results show that C-26 demethylation of CS is a degradative reaction that reduces the activity of CS as an active BR in *A. thaliana*.

We could not demonstrate the presence of 26-norBL in Arabidopsis plants. However, the enzyme solution prepared from Arabidopsis successfully catalyzed C-26 demethylation of BL to 26-norBL, suggesting that Arabidopsis is capable of C-26 demethylation of BL. Given that the endogenous level of BL is much lower (approximately 1/20-1/50) than that of CS in Arabidopsis,⁵ the endogenous level of 26-norBL produced from BL may be too low to be detected by the GC-MS/SIM method used in this study.

Previously, we reported an enzymatic conversion experiment that showed that C-26 demethylation of CS and BL to 26-norCS and 26-norBL does occur in *Phaseolus vulgaris*, and also in the lower plant *Marchantia polymorpha*.^{7,8} Coupled with the results of this study, this strongly indicates that the C-26 demethylation of the physiologically active BRs, CS and BL, is a common inactivating reaction that helps maintain the homeostatic level of active BRs in plants.

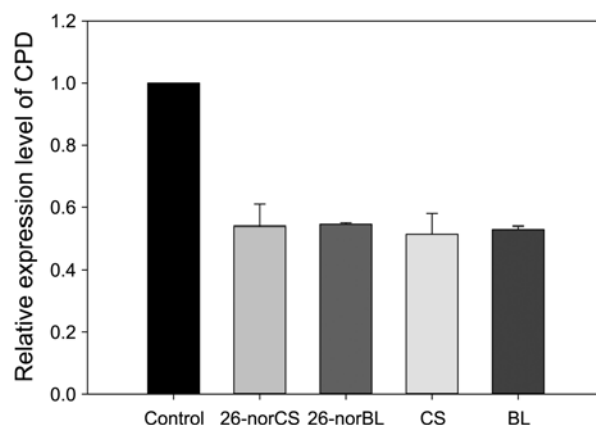


Figure 3. Comparison of CPD expression level in BR-treated Arabidopsis seedlings. Error bars indicate the average value of three independent experiments.

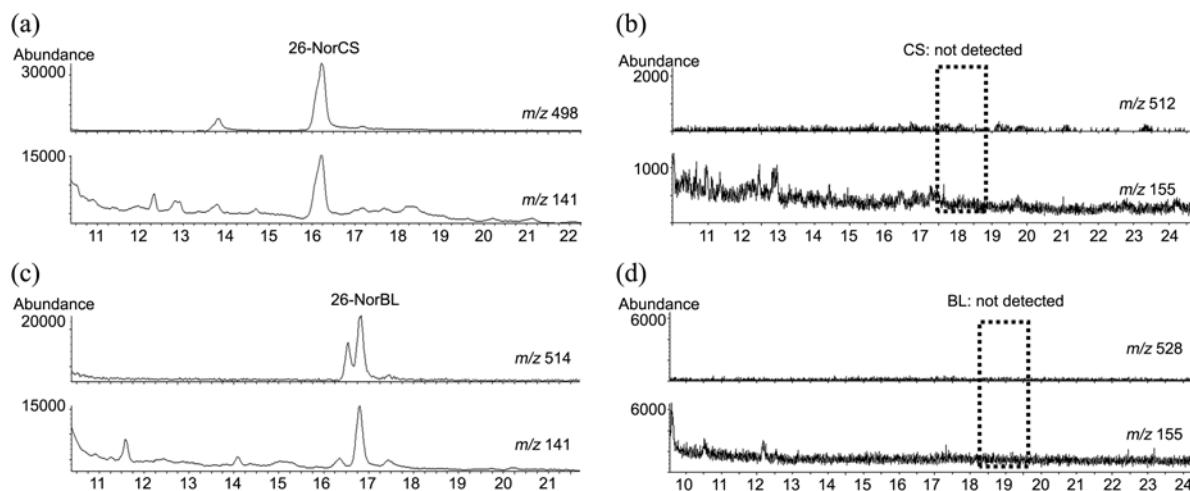


Figure 2. *In vitro* conversion of 26-norCS, 26-norBL, CS and BL by the enzyme solution prepared from *A. thaliana*. (a) Conversion of CS to 26-norCS is detected. (b) Conversion of 26-norCS to CS is not detected. (c) Conversion of BL to 26-norBL is detected. (d) Conversion of 26-norBL to BL is not detected.

Expression of *CPD* which encodes a C22-hydroxylase in BRs biosynthesis is known to be inhibited by CS and BL in *A. thaliana*. As shown in Figure 3, expression of *CPD* was also down-regulated by 26-norCS and 28-norBL in the plant. These indicate that endogenous levels of BRs are feedback regulated by 26-norCS and 26-norBL as well as CS and BL, which strictly controls biologically active BRs, CS and BL in *Arabidopsis* plants.

The C-26 demethylation of BRs is believed to be carried out *via* intermediates with C-26 functions either as alcohol, aldehyde, or carboxylic acid.¹¹ In support of this, the hydroxylation of C-26 has been shown to occur in tomato cell cultures, which convert 24-*epi*BL and 24-*epi*CS into 26-hydroxy-24-*epi*BL and 26-hydroxy-24-*epi*CS, respectively.⁸ In *Arabidopsis*, the *BAS1* gene was determined to encode cytochrome P450 (CYP734A1), which mediates the C-26 hydroxylation of BRs.¹² Recently, CYP734A orthologs from rice (CYP734A2, 4 and 6) have been identified as multi-functional enzymes that mediate C-26 hydroxylation and oxidation to generate C-26 aldehyde and C-26 carboxylate.¹³ In order to confirm the aforementioned reactions' role in C-26 demethylation, the oxidation reactions that form C-26 aldehyde and C-26 carboxylic acid, and the subsequent decarboxylation reaction that reduces C-26 methyl, should be further assessed by biochemical and molecular genetic studies with related mutants in *Arabidopsis*.

Experimental Section

Purification of 26-norCS in *A. thaliana*. A large quantity (30 kg) of *Arabidopsis thaliana* was extracted with 90% MeOH (30 L \times 3). The 80% MeOH soluble fraction was reduced to aqueous phase, and re-extracted with CHCl₃ (10 L \times 3). After drying *in vacuo*, the CHCl₃ soluble fraction was partitioned between *n*-hexane and 80% MeOH (5 L \times 3). The 80% MeOH soluble phase was concentrated, and re-partitioned between EtOAc and phosphate buffer (0.1 M sodium phosphate, pH 7.4). The EtOAc soluble fraction was subsequently collected, concentrated, and applied to a SiO₂ column (200 g). Elution was conducted with mixtures of MeOH-CHCl₃ with increasing MeOH concentration. In the rice lamina inclination bioassay, 5 to 8% MeOH in CHCl₃ revealed BR activity. The 5-8% MeOH fraction was subsequently loaded onto a Sephadex LH-20 column (2.5 \times 90 cm) and eluted with a mixture of CHCl₃:MeOH = 1:4. The fraction eluted between elution volume/bed volume = 0.60-0.70 was collected, then further purified *via* charcoal (10 g) column chromatography and eluted with mixtures of MeOH in CHCl₃. The fractions for MeOH:CHCl₃ = 1:1 and 1:2 were collected and purified *via* reverse-phase HPLC (Senshu-Pak C₁₈, 10 \times 150 mm) using aqueous methanol as a mobile phase (0-20 min: 45%, 20-40 min: gradient to 100%, 40-60 min: 100% methanol) at a flow rate of 2.5 mL/min. Fractions were collected every minute. HPLC fractions 9-11 and 13-15 were derivatized to be bismethaneboronate (BMB) with methaneboronic acid in pyridine (1 mg/2 mL), then analyzed *via* capillary GC-MS.

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

***In vitro* Enzyme Assay.** A crude enzyme solution was prepared from *Arabidopsis thaliana* (5 g) as the method previously reported.¹⁰ CS and BL were added to the enzyme solution, and incubated for 30 min at 37 °C. Afterward, the product was extracted with EtOAc, and loaded onto a C₁₈ cartridge eluted with aqueous MeOH (50, 60, 70, 80, 90 and 100% MeOH). The 70 and 80% MeOH fractions were collected and purified *via* reverse-phase HPLC as described above. The enzyme products were collected, and analyzed as BMB derivatives *via* capillary GC-MS.

Transcript Analysis. 7-day-old seedlings were transferred to D.W. containing 10⁻⁷ M of 26-norCS, 26-norBL, CS and BL and incubated for 3 h. Total RNA was extracted from seedlings using TRI reagent (Sigma) according to the manufacturer's instructions. For semi-quantitative RT-PCR, 2 μ g of total RNA was reverse-transcribed by M-MLV reverse transcriptase (Promega); 2 μ L of the RT product was employed as a PCR template and PCR were performed using RealTaq (RBC). PCR conditions were denaturation at 96 °C for 5 min, followed by 27 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min 40 sec. The gene-specific CPD primers were as follows: forward primer (5'-CCCGGGATGGCCTTCACCGCTTTTCT-3') and reverse primer (5'-GAGCTCTCAAGTAGCAAATCACGGCG-3'). As an internal control, UBQ5 cDNA was amplified using a forward primer (5'-GACCATAACCCTTGAGGTTGAATC-3') and a reverse primer (5'-AGAGAGAAAGAGAAGGATCGATC-3').

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