

# Carlactone is an endogenous biosynthetic precursor for strigolactones

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**Strigolactones (SLs) are a class of terpenoid plant hormones that regulate shoot branching as well as being known as root-derived signals for symbiosis and parasitism. SL has tricyclic-lactone (ABC-ring) and methyl butenolide (D-ring), and they are connected through an enol ether bridge. Recently, a putative biosynthetic intermediate called carlactone (CL), of which carbon skeleton is in part similar to those of SLs, was identified by biochemical analysis of three biosynthetic enzymes, DWARF27, CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), and CCD8 in vitro. However, CL has never been identified from plant tissues, and the conversion of CL to SLs has not been proven in vivo. To address these questions, we chemically synthesized <sup>13</sup>C-labeled CL. We show that <sup>13</sup>C-labeled CL is converted to (–)-[<sup>13</sup>C]-2′-epi-5-deoxystrigol ((–)-2′-epi-5DS) and [<sup>13</sup>C]-orobanchol, endogenous SLs in rice, in the *dwarf10* mutant, which is defective in CCD8. In addition, we successfully identified endogenous CL by using liquid chromatography-quadrupole/time-of-flight tandem mass spectrometry in rice and *Arabidopsis*. Furthermore, we determined the absolute stereochemistry of endogenous CL to be (11*R*)-configuration, which is the same as that of (–)-2′-epi-5DS at the corresponding position. Feeding experiments showed that only the (11*R*)-isomer of CL, but not the (11*S*)-isomer, was converted to (–)-2′-epi-5DS in vivo. Taken together, our data provide conclusive evidence that CL is an endogenous SL precursor that is stereospecifically recognized in the biosynthesis pathway.**

cytochrome P450 | phosphate deficiency

**S**trigolactones (SLs) are a group of terpenoid lactones that were originally identified as seed germination stimulants for root parasitic plants (1). They were later characterized as root-derived signals for arbuscular mycorrhizal fungi that supply inorganic nutrients to the host plants (2). In 2008, it was reported that SLs act as endogenous hormones, or their biosynthetic precursors, that regulate shoot branching (3, 4). Recent papers reported that SLs control more diverse developmental processes, such as root development, leaf senescence, secondary growth of the stem, and so on (5–7).

Until recently, little was known about the SL biosynthetic pathway. In 2005, it was reported that SL might be biosynthesized via the carotenoid pathway based on the experiment using a carotenoid biosynthesis inhibitor, fluridone, and some carotenoid metabolism mutants (8). Later studies demonstrated the contribution of two carotenoid cleavage dioxygenases (CCD7 and CCD8) to SL biosynthesis (3, 4). One cytochrome P450, MORE AXILLARY GROWTH1 (MAX1), was also shown to act in SL biosynthesis (9). Later, another SL-deficient mutant of rice, *dwarf27* (*d27*), was characterized (10). The *D27* gene was found to encode a member of a novel class of Fe-containing proteins and to localize to chloroplasts.

Recently, a possible SL biosynthetic intermediate called carlactone (CL), which has a SL-like carbon skeleton, was identified as a product of sequential reactions by three biosynthetic enzymes, D27, CCD7, and CCD8 in vitro (Fig. 1) (11). In this report, the authors demonstrated that D27 catalyzes the isomerization at C-9

position of all-*trans*-β-carotene, and the product, 9-*cis*-β-carotene, is further converted to CL via sequential reactions by CCD7 and CCD8. Before the discovery of CL, SLs were predicted to be biosynthesized by coupling of the carotenoid-derived ABC-ring portion with the D-ring butenolide part (8, 12). However, CL was shown to be enzymatically synthesized from 9-*cis*-β-apo-10′-carotenal through the intramolecular rearrangement catalyzed by CCD8, suggesting that one SL molecule is derived from one β-carotene molecule (Fig. 1). Moreover, CL was shown to inhibit shoot branching of rice SL biosynthetic mutants, such as *d10* and *d27*, and stimulated seed germination of *Striga hermonthica*, suggesting that CL exerts SL activities by itself or as a biosynthetic intermediate of SLs (11). Very recently, Scaffidi et al. reported the chemical synthesis of CL and its biological activity in *Arabidopsis* (13). They showed that CL suppresses shoot branching of *Arabidopsis* via a mechanism dependent on the cytochrome P450 MAX1, indicating that MAX1 is a downstream component of CL biosynthesis (Fig. 1) (13).

Although CL is a possible biosynthetic precursor for SLs, this compound has not been detected from plant tissues as an endogenous compound (11). Moreover, it has yet to be clarified whether CL is converted to SLs in vivo. To address these questions, we synthesized <sup>13</sup>C-labeled CL and carried out feeding experiments using a putative rice CL-deficient mutant, *dwarf10* (*d10*). We also attempted to identify endogenous CL from rice and *Arabidopsis* using liquid chromatography-quadrupole/time-of-flight tandem mass spectrometry (LC-MS/MS). As a result, we successfully observed the conversion of <sup>13</sup>C-labeled CL to

## Significance

**Strigolactones (SLs) were initially characterized as root-derived signals for parasitic and symbiotic interactions with root parasitic plants and arbuscular mycorrhizal fungi, respectively. SLs were later shown to act as endogenous hormones that regulate shoot branching. Carlactone (CL) was identified as a product of three SL biosynthetic enzymes in vitro, and therefore a putative biosynthetic precursor for SLs. However, it was neither detected from plant tissues, nor was the conversion of CL to SL demonstrated in vivo. In this paper, we show that <sup>13</sup>C-labeled CL is converted to SLs in vivo, and that endogenous CL is successfully identified from rice and *Arabidopsis*. These results demonstrate that CL is a true biosynthetic precursor for SLs.**

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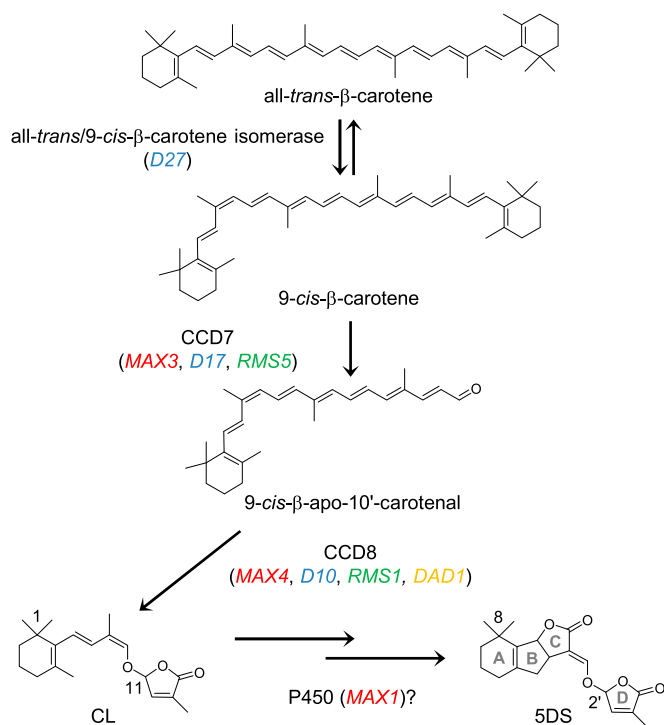
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**Fig. 1.** Predicted biosynthetic pathway of SLs. CL is produced from all-*trans*- $\beta$ -carotene by three enzymes, D27, CCD7, and CCD8. D27 catalyzes the isomerization reaction of all-*trans*- $\beta$ -carotene at C-9 position. CL would be oxidized by some oxidation enzymes such as the cytochrome P450 monooxygenase MAX1 and then converted to 5DS. Red, blue, green, and orange characters indicate genes of *Arabidopsis*, rice, pea, and petunia, respectively. Single arrows show conversions catalyzed by a single enzyme, and double arrows denote conversions possibly catalyzed by more than two enzymes.

(-)-[ $^{13}\text{C}$ ]-2'-*epi*-5-deoxystrigol ((-)-2'-*epi*-5DS) and [ $^{13}\text{C}$ ]-orobanchol in rice. Moreover, we could detect endogenous CL in rice and *Arabidopsis* root extracts. In addition, we determined the absolute stereochemistry of endogenous CL to be (11*R*)-configuration. Furthermore, we used  $^{13}\text{C}$ -labeled CL as an internal standard to quantify endogenous CL in rice and *Arabidopsis*; we assessed whether endogenous CL levels are affected by phosphate (Pi) deficiency like endogenous SL levels.

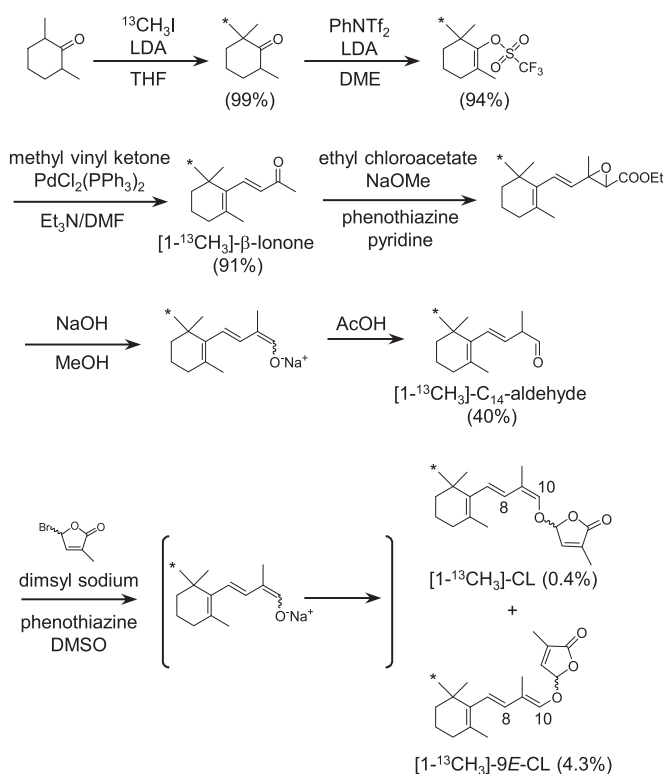
## Results

**Chemical Synthesis of Stable Isotope-Labeled CL.** To chemically prepare [ $^{13}\text{C}$ ]-CL for feeding experiments (Fig. 2), the  $^{13}\text{C}$  label was incorporated at 2- $\text{CH}_3$  of 2,2,6-trimethylcyclohexanone by methylation of 2,6-dimethylcyclohexanone with  $^{13}\text{C}$ -methyl iodide and lithium diisopropylamide. The  $^{13}\text{C}$ -labeled cyclohexanone was converted to cyclohexenyl triflate, which was subjected to the Heck reaction with methyl vinyl ketone to afford [ $^{13}\text{C}$ ]- $\beta$ -ionone (14). This was converted to  $\text{C}_{14}$ -aldehyde according to literature procedure (15). The sodium enolate was generated from the  $\text{C}_{14}$ -aldehyde with dimethyl sulfide in dimethyl sulfoxide (16), which was followed by *O*-alkylation with bromo-2'-*epi*-5DS to provide [ $^{13}\text{C}$ ]-CL and its (9*E*) geometric isomer in a ratio ~1:10 (Fig. 2). The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra of  $^{13}\text{C}$ -labeled CL were consistent with those reported previously (11), except for the splitting of signals of  $\text{CH}_3$ -16 and  $\text{CH}_3$ -17 protons and of C-1, C-3, and C-5 carbons due to the incorporation of a  $^{13}\text{C}$  label into the molecule. The 9*E*-geometry of the isomer was confirmed by an NOE correlation between H-8 (5.88 ppm) and H-10 (6.42 ppm) in the NOESY spectrum. Although a 4.3-fold increase was achieved in the yield of *O*-alkylation of  $\text{C}_{14}$ -enolate by adding phenothiazine to the reaction mixture, the overall yield was quite low. Nevertheless, racemic [ $^{13}\text{C}$ ]-CL was synthesized

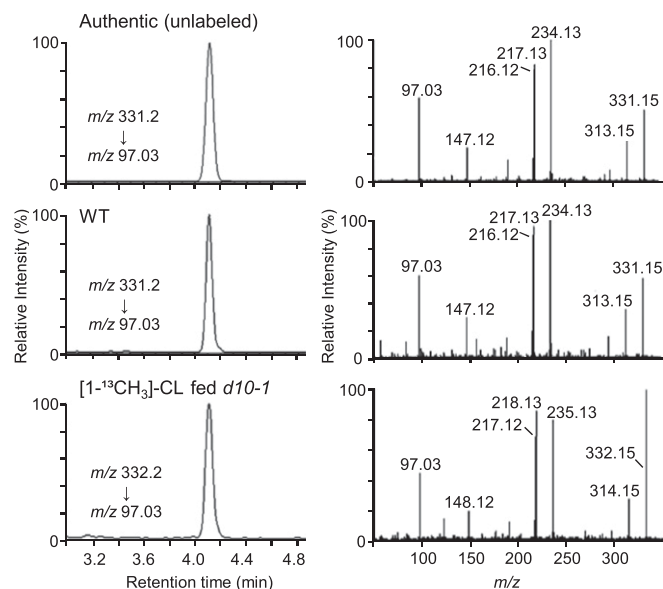
and subjected to optical resolution by semipreparative chiral HPLC to afford a pair of enantiomers in sufficient quantities for feeding experiments. Unlabeled CL was synthesized from commercially available  $\beta$ -ionone and optically resolved as above.

**Feeding Experiments of [ $^{13}\text{C}$ ]-CL to the Rice *d10-1* Mutant.** Using synthetic *rac*-[ $^{13}\text{C}$ ]-CL, we carried out feeding experiments to examine whether CL is converted to SLs *in vivo*. We used the rice *d10-1* mutant, which is defective in the *CCD8* gene (17). SLs were undetectable in the *d10-1* mutant (4). CCD8 catalyzes the intramolecular rearrangement of 9-*cis*- $\beta$ -apo-10'-carotenol to yield CL *in vitro* (11). Therefore, we can expect that the *d10-1* mutant does not produce CL. The *d10-1* mutant would be a suitable plant material for feeding labeled CL to see its metabolism because the labeled substrate would not be diluted by endogenous unlabeled CL. A hydroponic culture system was used for cultivating *d10-1* plants, and *rac*-[ $^{13}\text{C}$ ]-CL was added to the hydroponic culture media at 1  $\mu\text{M}$ . As a negative control, the *d10-1* mutant was grown under the same condition without feeding *rac*-[ $^{13}\text{C}$ ]-CL. WT Shiokari cultivar without any treatment was also grown. After feeding for 2 d, we analyzed 2'-*epi*-5DS, which is an endogenous SL in the rice Shiokari cultivar (4). The root exudates of *d10-1* were analyzed by LC-MS/MS, and we could successfully detect the peak of 2'-*epi*-5DS whose *m/z* is increased by one mass unit in *rac*-[ $^{13}\text{C}$ ]-CL-fed samples compared with an unlabeled authentic standard (Fig. 3). In the full-scan spectra, almost all fragment ions except for *m/z* 97.03, which is derived from the D-ring part, were increased by one mass unit compared with an unlabeled authentic standard or 2'-*epi*-5DS from WT plants. These results indicate the bioconversion of *rac*-[ $^{13}\text{C}$ ]-CL to [ $^{13}\text{C}$ ]-2'-*epi*-5DS by the *d10-1* mutant. In the negative control analysis, we could not detect 2'-*epi*-5DS.

Next, we determined the absolute stereochemistry of the endogenously converted [ $^{13}\text{C}$ ]-2'-*epi*-5DS using chiral LC-MS/MS. For this experiment, we analyzed the root extract to examine



**Fig. 2.** Chemical synthesis of [ $^{13}\text{C}$ ]-CL. Asterisks indicate the position of  $^{13}\text{C}$ . Details are described in *SI Materials and Methods*.



**Fig. 3.** LC-MS/MS analysis of 2'-*epi*-5DS from *d10-1* root exudates after feeding  $[1-^{13}\text{C}]$ -CL. Selected reaction monitoring (Left) and full-scan spectra of fragment ions (Right) are shown.

whether the conversion occurred truly *in vivo*. 5DS has four stereoisomers (Fig. S1), and it was reported that the rice Nipponbare cultivar only produces (–)-2'-*epi*-5DS (18). Although we have previously shown that the Shiokari cultivar produces 2'-*epi*-5DS, its absolute stereochemistry has not been determined (4). As shown in Fig. 4A, four isomers of 5DS were eluted at different retention times in our LC condition using a reverse phase chiral column. By the comparison of its retention time with those of authentic stereoisomers, we could confirm that the Shiokari cultivar also produces only (–)-2'-*epi*-5DS as an endogenous isomer (Fig. 4A). Importantly, the stereoisomer of 2'-*epi*-5DS endogenously converted from *rac*-CL was determined to be (–)-2'-*epi*-5DS, which is the same as the endogenous one (Fig. 4A).

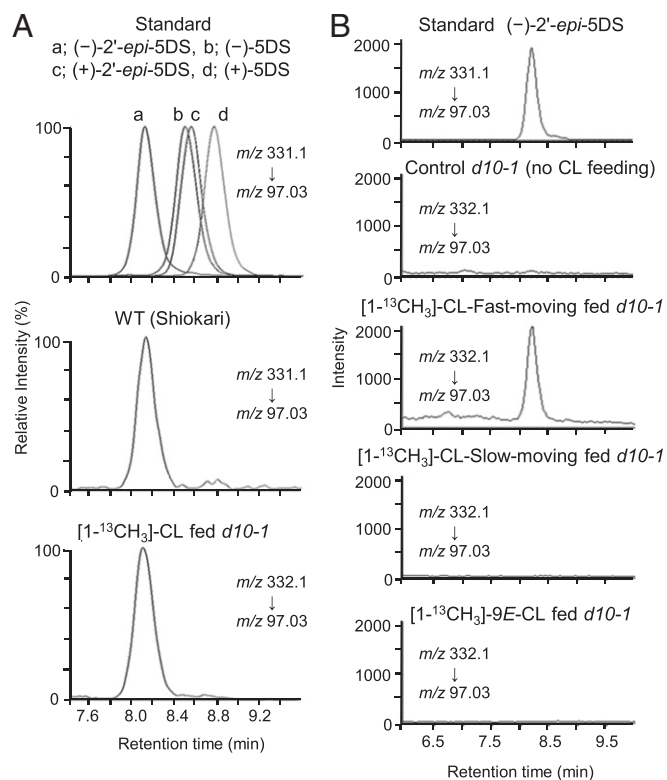
In the feeding experiments, we used a racemic mixture of  $[1-^{13}\text{C}]$ -CL. However, only one stereoisomer of 5DS was synthesized *in vivo*, suggesting that the enzyme(s) involved in the conversion(s) has a stereospecificity. To examine this stereospecificity in SL biosynthesis, each enantiomer of  $[1-^{13}\text{C}]$ -CL was fed to the *d10-1* mutant at 1  $\mu\text{M}$ . We call the isomer eluted faster from the reverse phase chiral column “CL-fast-moving” and the other isomer “CL-slow-moving.”  $[1-^{13}\text{C}]$ -9*E*-CL, which was obtained as a by-product in the chemical synthesis of  $[1-^{13}\text{C}]$ -CL, was also examined to check whether this chemical can be a precursor for (–)-2'-*epi*-5DS (Fig. 2). As a result, only one enantiomer,  $[1-^{13}\text{C}]$ -CL-fast-moving, was converted to (–)- $[8-^{13}\text{C}]$ -2'-*epi*-5DS, suggesting that the CL-metabolizing enzyme recognizes only one stereoisomer of CL (Fig. 4B). To examine the  $[^{13}\text{C}]$  incorporation from  $[1-^{13}\text{C}]$ -CL into other SL molecules, we carried out a feeding experiments using the rice *d10-2* mutant (17), whose background is Nipponbare, because Nipponbare is known to produce (–)-orobanchol in addition to (–)-2'-*epi*-5DS (18). As a result, we could detect  $[8-^{13}\text{C}]$ -orobanchol in the root exudate of *d10-2* seedlings after feeding  $[1-^{13}\text{C}]$ -CL-fast-moving (Fig. S2). These results provide direct evidence that CL can be converted to SLs in rice in a stereospecific manner.

**Identification of Endogenous CL Using LC-MS/MS.** Alder et al. could not detect CL in plants (11). We attempted to detect CL from rice root extracts to assess whether CL is an endogenous precursor for SLs. We firstly selected the rice SL-insensitive mutant *d14-1* (19) as a plant material, because a high level of SL is

accumulated in this mutant, possibly due to feedback regulation (19). The extract of roots was purified through several column chromatography techniques and the purified sample was analyzed by LC-MS/MS using synthetic *rac*- $[1-^{13}\text{C}]$ -CL as an internal standard. We selected  $[\text{M}+\text{H}]^+$  ( $m/z$  303 and  $m/z$  304 for unlabeled and *rac*- $[1-^{13}\text{C}]$ -CL, respectively) as parent ions on quadrupole mass spectrometry, and detected  $[\text{M}+\text{H}-206]^+$  ( $m/z$  97.03 for both unlabeled and *rac*- $[1-^{13}\text{C}]$ -CL) as a fragment ion on time-of-flight mass spectrometry after collision-induced dissociation. We could detect one peak whose retention time is identical with that of the authentic standard of *rac*-CL (Fig. 5A). Full-scan spectra of fragment ions (Fig. 5A) and selected reaction monitoring using major fragment ions (Fig. S3) confirmed the identity of this endogenous compound.

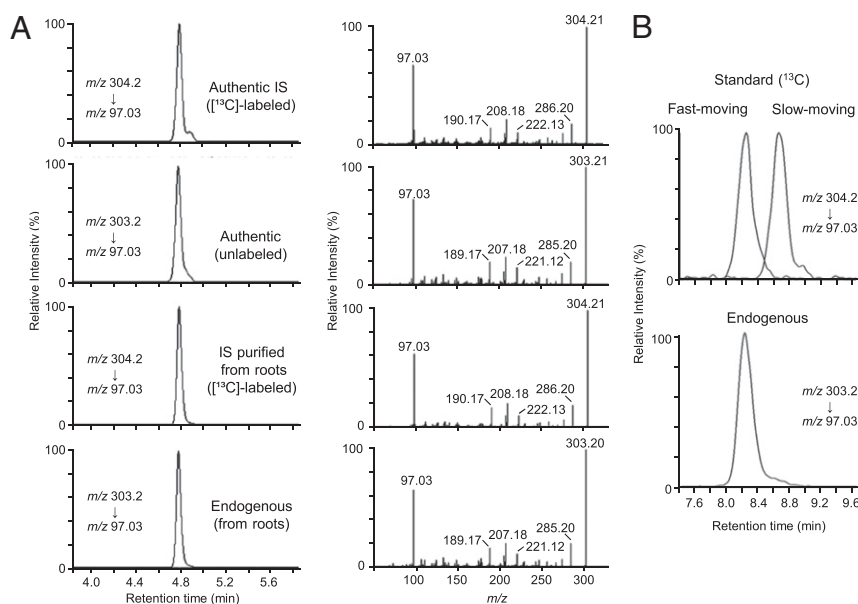
Next, we analyzed stereochemistry of endogenous CL using chiral LC-MS/MS. By the comparison of the retention time of endogenous CL with that of authentic standards, we determined endogenous CL to be CL-fast-moving, which is the same one that was converted to (–)-2'-*epi*-5DS and orobanchol in the feeding experiment (Fig. 5B).

To examine whether CL exists in other plant species, we analyzed endogenous CL in *Arabidopsis*. As a plant material, we used the *atd14-2* mutant, which is defective in the *Arabidopsis* ortholog of rice *D14* (Fig. S4). Endogenous CL was successfully identified from root extracts of *atd14-2* by LC-MS/MS (Fig. S5). We also found that the stereochemistry of endogenous CL in *Arabidopsis* is the same as that in rice (Fig. S6). These results indicate that CL-fast-moving is a common precursor for SL in rice and *Arabidopsis*.



**Fig. 4.** Determination of the stereochemistry of 5DS. (A) Chiral LC-MS/MS analysis of stereoisomers of 5DS in root extracts after feeding *rac*- $[1-^{13}\text{C}]$ -CL. Selected reaction monitoring of synthetic standards of 5DS stereoisomers, (–)-2'-*epi*-5DS from WT root extracts, and (–)-2'-*epi*-5DS from *d10-1* root extracts after feeding *rac*- $[1-^{13}\text{C}]$ -CL. (B) Chiral LC-MS/MS analysis of (–)-2'-*epi*-5DS from root extracts after feeding each enantiomer of  $[1-^{13}\text{C}]$ -CL or  $[1-^{13}\text{C}]$ -9*E*-CL.





**Fig. 5.** Identification of endogenous CL in plants. (A) LC-MS/MS analysis of CL from root extracts of rice *d14-1*. Selected reaction monitoring (*Left*) and full-scan spectra of fragment ions (*Right*) of authentic [ $1-^{13}\text{C}$ ]-CL, unlabeled authentic standard, [ $1-^{13}\text{C}$ ]-CL purified from root extracts, and endogenous CL. (B) Chiral LC-MS/MS analysis of CL from root extracts of rice *d14-1*. Selected reaction monitoring of authentic standards of two enantiomers of [ $1-^{13}\text{C}$ ]-CL, and endogenous CL.

**Determination of the Absolute Stereochemistry of CL.** The results described so far indicate that only one enantiomer of CL is present in rice and *Arabidopsis*, and this isomer can be converted to (–)-2′-*epi*-5DS and orobanchol in rice. To clarify the absolute stereochemistry of natural CL, we determined the absolute configuration of the synthetic isomers at C-11. We first observed circular dichroism (CD) spectra of the CL-fast-moving and CL-slow-moving enantiomers. In the case of SL stereoisomers, the absolute configuration at C-2′ position in the D-ring was determined by the cotton effect around 260 nm (20, 21). However, the CD spectra of CL were not similar to those of SLs (Fig. S7). Thus, we could not use this method to determine the absolute stereochemistry of CL. We then determined the stereochemistry after derivatization of CL as a Diels-Alder (DA) adduct with cyclopentadiene. As shown in Fig. 6A, coupling of the (5*S*)- or (5*R*)-modified D-ring portion with the  $\text{C}_{14}$ -aldehyde gave each enantiomer of DA adduct-type CL with fixed stereochemistry at C-11 (22). Then, CL-fast-moving, which is the endogenous isomer, and CL-slow-moving were separately reacted with cyclopentadiene using Lewis acid to yield DA adducts (Fig. 6A) (23). LC-MS analysis of these reaction products compared with each enantiomer of the synthetic standard allowed us to determine the configuration at the C-11 position of CL-fast-moving and CL-slow-moving to be (*R*) and (*S*), respectively (Fig. 6B). These results demonstrate that endogenous CL has (1*R*)-configuration, which is the same as that of the corresponding position (C-2′) of (–)-2′-*epi*-5DS (Fig. 6C).

**Quantitative Analysis of Endogenous Levels of (*R*)-CL.** We quantified endogenous levels of (*R*)-CL in rice by LC-MS/MS using *rac*-[ $1-^{13}\text{C}$ ]-CL as an internal standard. Previous studies have shown that SL levels in roots and root exudates are highly elevated by phosphate (Pi) or nitrate starvation in various plant species (4, 24–27). To address whether (*R*)-CL levels are also affected by Pi-deficiency, we quantified endogenous (*R*)-CL under +Pi and –Pi conditions in WT, *d10-1* and *d14-1* rice seedlings. Endogenous (*R*)-CL was detected in WT root extracts, but not in *d10-1* root extracts (Fig. 7A). (*R*)-CL levels were much higher in the *d14-1* mutant than in WT plants, as was the case with SL levels (Fig. 7A) (19). Interestingly, Pi-deficiency did not increase the levels of (*R*)-CL in WT and *d14-1* root extracts, unlike the case with SL ((–)-2′-*epi*-5DS) (Fig. 7A).

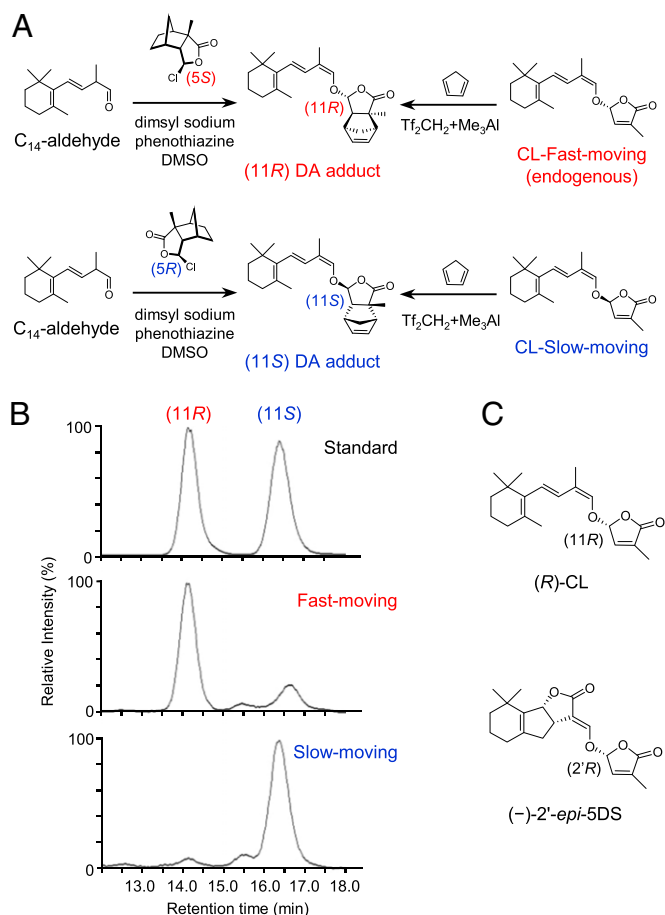
We also quantified endogenous (*R*)-CL levels in *Arabidopsis* roots. We grew WT, *max4-8* (defective in CCD8), *max1-4*

(defective in a cytochrome P450, Fig. S4), and *atd14-2* plants, and the root extracts were analyzed by LC-MS/MS. (*R*)-CL was undetectable in the *max4-8* mutant, whereas the *atd14-2* mutant accumulated a high level of (*R*)-CL as did the *d14-1* mutant of rice (Fig. 7B). Pi-deficiency did not affect the levels of (*R*)-CL so much also in *Arabidopsis* (Fig. 7B). *MAX1* is a possible downstream component of (*R*)-CL biosynthesis (Fig. 1), and (*R*)-CL is a candidate for the *MAX1* substrate (11, 13). Notably, *max1-4* accumulated extremely high levels of (*R*)-CL; ~5 times higher than *atd14-2* and 700 times higher than WT. These results suggest that (*R*)-CL is a substrate for *MAX1* (Fig. 7B).

To further examine the involvement of *MAX1* in (*R*)-CL metabolism, we carried out feeding experiments using *Arabidopsis*. In a previous report, orobanchol and orobanchol acetate were detected in *Arabidopsis* (28), whereas in our growth and analytical conditions, these known SLs were under the detection level limit. Instead, we found an SL-like molecule (SL-LIKE1) from *Arabidopsis* WT root extract (Fig. S8A). SL-LIKE1 showed a product ion peak characteristic of SLs at *m/z* 97.03, which is derived from the D-ring part (Fig. S8A). We could detect SL-LIKE1 also in the *atd14-2* mutant, but not in the *max1-4* mutant, suggesting the involvement of *MAX1* in SL-LIKE1 biosynthesis (Fig. S8B). We fractionated *atd14-2* root extracts by HPLC, and the fraction containing SL-LIKE1 stimulated the germination of *S. hermonthica* and *Orobancha minor* seeds (Fig. S8C). Then we analyzed the [ $^{13}\text{C}$ ] incorporation into this molecule from (*R*)-[ $1-^{13}\text{C}$ ]-CL. Although the *max1-4* mutant produces an extremely large amount of (*R*)-CL, endogenous (*R*)-CL in the *max1-4 max4-7* double mutant was undetectable (Fig. S8D). Moreover, (*R*)-[ $^{13}\text{C}$ ]-CL was converted into [ $^{13}\text{C}$ ]-SL-LIKE1 in the *max4-7* mutant, whereas [ $^{13}\text{C}$ ]-SL-LIKE1 was not detected in the *max1-4 max4-7* double mutant after feeding (*R*)-[ $1-^{13}\text{C}$ ]-CL (Fig. S8E). These results demonstrate the contribution of *MAX1* to the (*R*)-CL metabolism pathway in *Arabidopsis*.

## Discussion

CL was found as a putative intermediate in SL biosynthesis through *in vitro* experiments using recombinant enzymes. However, neither the occurrence of CL in plants has been proved nor the conversion of CL to SLs in plants has been demonstrated in a previous study (11). Here, we report the bioconversion of chemically synthesized [ $^{13}\text{C}$ ]-labeled CL to (–)-[ $^{13}\text{C}$ ]-2′-*epi*-5DS and [ $^{13}\text{C}$ ]-orobanchol, endogenous SLs in rice, and the detection of endogenous CL from rice and *Arabidopsis*. Moreover, the



**Fig. 6.** Determination of the absolute stereochemistry of CL. (A) Synthetic scheme of DA adduct-type CL. As authentic standards, two enantiomers of DA adduct-type CL were prepared from  $C_{14}$  aldehyde by coupling with each stereoisomer (5*S* or 5*R*) of the modified butenolide portion. CL-fast-moving and CL-slow-moving were separately reacted with cyclopentadiene by DA reaction to afford the cycloadducts. (B) Chiral-LC-MS analysis of each enantiomer of DA adduct-type CL (standard) and DA adduct-type CL synthesized from CL-fast-moving or CL-slow-moving. (C) Chemical structures of (*R*)-CL and (–)-2′-epi-5DS.

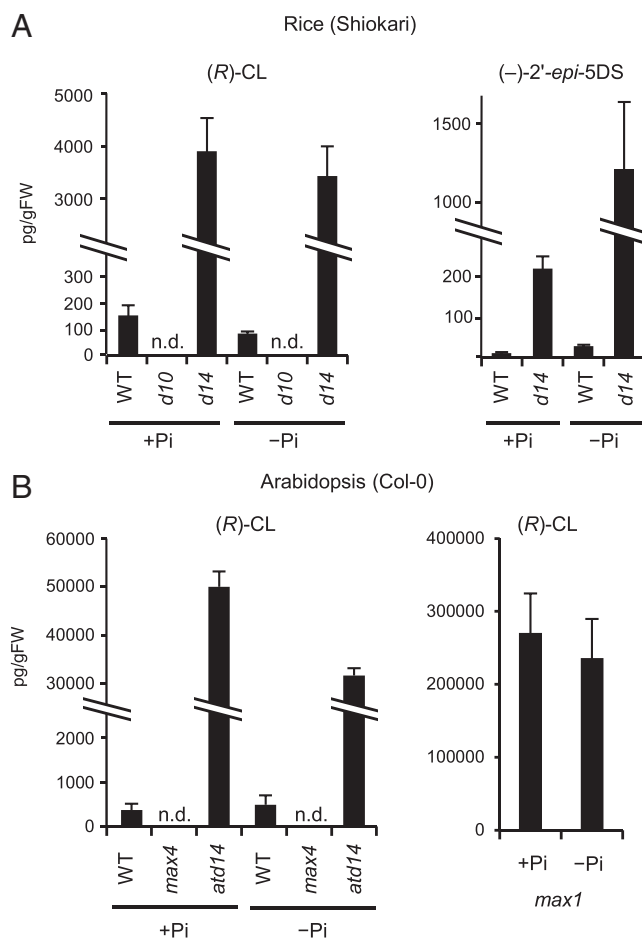
absolute stereochemistry of the butenolide moiety of endogenous CL was determined to be the same as that of endogenous (–)-2′-epi-5DS. These data conclusively demonstrate that (*R*)-CL is an endogenous biosynthetic precursor for SLs.

Interestingly, in our feeding experiments, only one stereoisomer of CL (CL-fast-moving) was converted to (–)-2′-epi-5DS as revealed by chiral LC-MS/MS analysis (Fig. 4*B*). In addition, only this stereoisomer was detected as endogenous CL both in rice and in *Arabidopsis* (Fig. 5*B* and Fig. S6). Chemical synthesis and chiral LC-MS analysis of DA adduct-type CL enabled us to determine the absolute stereochemistry of CL-fast-moving to be (11*R*)-configuration, which is the same as that of the corresponding position (C-2′) of (–)-2′-epi-5DS (Fig. 6). The stereochemistry of C-2′ position has been determined to be (*R*) in all natural SLs reported so far; natural 5DS is (+)-5DS or (–)-2′-epi-5DS (Fig. 6*C* and Fig. S1) (18, 29). Although some SLs have been detected in *Arabidopsis*, their absolute stereochemistry has not been determined (28). We could detect the (11*R*) isomer of CL from *Arabidopsis*, providing the indirect evidence that *Arabidopsis* also produces (2′*R*) type SLs like other plant species (Fig. S6).

Our quantitative analysis of endogenous (*R*)-CL revealed that (*R*)-CL levels in the *d14-1* mutant of rice and the *atd14* mutant of *Arabidopsis*, both of which are proposed SL perception mutants

(19, 30–32), are highly elevated in comparison with WT plants, possibly due to feedback regulation (Fig. 7*A*). *rac*-CL was reported to weakly suppress hypocotyl elongation in a *MAX1*-independent manner (13). However, it does not inhibit shoot branching without *MAX1* function, suggesting that (*R*)-CL is a biologically inactive precursor of SLs at least for the regulation of shoot branching (13). In the case of *gid1*, a mutant of a soluble gibberellin (GA) receptor, the accumulation of only the active form of GA, such as  $GA_1$ , was observed (33). Therefore, the accumulation of biologically inactive (*R*)-CL in the *d14-1* and *atd14-2* mutants implies that a step (s) upstream of (*R*)-CL synthesis is highly elevated in these mutants through feedback regulation.

Interestingly, the levels of (*R*)-CL in rice and *Arabidopsis* were not elevated under Pi-deficient conditions, whereas the levels of (–)-2′-epi-5DS were highly elevated in rice as reported previously (Fig. 7) (4, 24–27). Rather, we could see a tendency that (*R*)-CL levels were slightly decreased by Pi deficiency (Fig. 7). In *Arabidopsis*, the levels of SLs were also elevated by Pi starvation as seen in rice (28). In contrast to the slight decrease in (*R*)-CL levels under –Pi condition, expression levels of some SL biosynthetic genes, including the upstream components of (*R*)-CL synthesis, were up-regulated by Pi deficiency in rice (27). This suggests that (*R*)-CL synthesis might be elevated under Pi-deficient conditions. We can speculate that Pi deficiency more highly up-regulates the pathway downstream of (*R*)-CL synthesis. It is



**Fig. 7.** Quantitative analysis of (*R*)-CL and (–)-2′-epi-5DS in plants. (A) Endogenous levels of (*R*)-CL and (–)-2′-epi-5DS in root extracts of rice WT, *d10-1*, and *d14-1* in the presence or absence of Pi. N.d., not detected due to low abundance. Data are the means  $\pm$  SD ( $n = 3$ ). (B) (*R*)-CL levels in root extracts of *Arabidopsis* WT Col-0, *max4-8*, *atd14-2*, and *max1-4*. N.d., not detected due to low abundance. Data are the means  $\pm$  SD ( $n = 3$ ).

noteworthy that the endogenous amount of (*R*)-CL in the presence of Pi is about 15 times higher than that of (–)-2'-*epi*-5DS (Fig. 7A). Taken together, our results suggest that plants store (*R*)-CL or (*R*)-CL metabolite(s) under normal growth conditions, and when plants need SLs, such as under low nutrient conditions, SLs are synthesized from (*R*)-CL or (*R*)-CL metabolite(s) and control the plant architecture, including shoot branching. Our data imply that there is (are) key step(s) for controlling the endogenous SL levels in the pathway between (*R*)-CL and SL in response to nutrient conditions.

(*R*)-CL needs to be further oxidized to be finally converted to SLs. Grafting experiments, using *Arabidopsis max1*, as a rootstock, and *max3* or *max4* as a scion, resulted in a restored shoot branching phenotype (34). These observations suggest that *MAX1* is a downstream component of *MAX3* and *MAX4*, and that a possible substrate for *MAX1* is (*R*)-CL (Fig. 1). As mentioned above, a recent report that *rac*-CL suppresses *Arabidopsis* shoot branching in a *MAX1*-dependent manner supports this hypothesis (13). Our quantitative analysis of endogenous (*R*)-CL revealed that the *max1-4* mutant accumulates an exceptionally high level of (*R*)-CL. Furthermore, we demonstrated that (*R*)-[1-<sup>13</sup>C<sub>3</sub>]-CL is converted into SL-LIKE1 in *Arabidopsis* in a *MAX1*-dependent manner, providing the direct evidence for the involvement of *MAX1* in the (*R*)-CL metabolism pathway (Fig. S8).

In conclusion, our results demonstrate that (*R*)-CL is an endogenous biosynthetic precursor of SLs in rice and *Arabidopsis*. Considering the structures of CL and SLs, at least two positions

of CL should be oxidized to be converted into SLs, indicating that there is one unidentified enzyme or more besides *MAX1* (Fig. 1). Our discovery will assist the understanding of the whole picture of the SL biosynthetic pathway and its regulatory mechanisms.

## Materials and Methods

**Plant Materials and Growth Conditions.** We used rice cultivar (*Oryza sativa* L. cv. Shiohari or Nipponbare) as the WT, and tillering dwarf mutants, *d10-1* and *d14-1*, in the Shiohari background, and *d10-2* in the Nipponbare background in this study (17, 19). We used *Arabidopsis* ecotype Col-0 as the WT and *max1-4* (SAIL\_25A05, Fig. S4), *max4-8* (SALK\_02750) (4), *max4-7* (SALK\_082552) (4), and *atd14-2* mutants. The *atd14-2* mutant was obtained from a TILLING project (<http://tilling.fhcr.org/>), and the mutation is shown in Fig. S4. The *atd14-2* mutant was used for experiments after a backcross with Col-0. Rice and *Arabidopsis* plants were grown hydroponically as described previously (4). Details of growth conditions and feeding experiments are described in *SI Materials and Methods*.

**LC-MS/MS Analysis and Chemical Synthesis.** LC-MS/MS analysis of 5DS and CL were performed as described in *SI Materials and Methods*. Methods of chemical synthesis are described in detail in *SI Materials and Methods*.

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