Strigolactones (SLs) are plant hormones that regulate shoot branching and diverse developmental processes. They are biosynthesized from carotenoid molecules via a key biosynthetic precursor called carlactone (CL) and its carboxylated analog, carlactonoic acid (CLA). We have previously identified the methyl esterified derivative of CLA, methyl carlactonoate (MeCLA), as an endogenous SL-like molecule in Arabidopsis. Neither CL nor CLA could interact with the receptor protein, Arabidopsis DWARF14 (AtD14), in vitro, while MeCLA could, suggesting that the methylation step of CLA is critical to convert a biologically inactive precursor to a bioactive compound in the shoot branching inhibition pathway. Here, we show that a member of the SABATH protein family ([Ar4g36470] efficiently catalyzes methyl esterification of CLA using S-adensyl-L-methionine (SAM) as a methyl donor. We named this enzyme CLAMT for CLA methyltransferase. The Arabidopsis loss-of-function clamt mutant accumulated CLA and had substantially reduced MeCLA content compared with wild type (WT), showing that CLAMT is the main enzyme that catalyzes CLA methylation in Arabidopsis. The clamt mutant displayed an increased branching phenotype, yet the branch number was less than that of severe SL biosynthetic mutants. Exogenously applied MeCLA, but not CLA, restored the branching phenotype of the clamt mutant. In addition, grafting experiments using the clamt and other SL biosynthetic mutants suggest that CL and CLA are transmissible from root to shoot. Taken together, our results demonstrate a significant role of CLAMT in the shoot branching inhibition pathway in Arabidopsis.

Significance

Strigolactones (SLs) are a group of apocarotenoid hormones, which regulate shoot branching and other diverse developmental processes in plants. The major bioactive form(s) of SLs as endogenous hormones has not yet been clarified. Here, we identify an Arabidopsis methyltransferase, CLAMT, responsible for the conversion of an inactive precursor to a biologically active SL that can interact with the SL receptor in vitro. Reverse genetic analysis showed that this enzyme plays an essential role in inhibiting shoot branching. This mutant also contributed to specifying the SL-related metabolites that could move from root to shoot in grafting experiments. Our work has identified a key enzyme necessary for the production of the bioactive form(s) of SLs.
enzyme activities of 13 SABATH proteins that were randomly selected from each clade of the phylogenetic tree or that were expressed in axillary buds in the preliminary RT-PCR analysis (SI Appendix, Fig. S1). Each of these was cloned into a protein expression vector, pET47b, and expressed in Escherichia coli as a His-tag fusion protein. The methylation activity against CLA was tested using the soluble protein fraction of each transformant, and we found that the cell lysate expressing At4g36470 efficiently catalyzed CLA methylation using SAM as a methyl donor (Fig. 2 and SI Appendix, Fig. S2). To further validate the biochemical function of At4g36470, we reconstituted the Arabidopsis SL biosynthetic pathway in Nicotiana benthamiana. Production of MeCLA was observed in N. benthamiana leaves coexpressing At4g36470 and the CLA biosynthetic enzymes (AtD27, MAX3/CCD7, MAX4/CCD8, and MAX1) (SI Appendix, Fig. S3). Phylogenetic analysis of all 24 SABATH family proteins in Arabidopsis suggests that there are no closely related homologs of At4g36470 (SI Appendix, Fig. S1). We named At4g36470 CLA methyltransferase (CLAMT) and further analyzed its physiological function using loss-of-function mutants.

Characterization of the Arabidopsis clamt Knockout Mutants and Their Phenotypic Analysis. We identified two independent alleles of the Arabidopsis clamt mutant in the Institute of Physical and Chemical Research (Japan) RIKEN Arabidopsis transposon-tagged collection in the Nossen (No-0) background (line RATM11-1868-1, clamt-1 and RATM53-2997-1, clamt-2) (SI Appendix, Fig. S4A) (20). In both of these alleles, the CLAMT transcript levels were significantly reduced compared with wild-type (WT) No-0 (SI Appendix, Fig. S4B). In order to investigate the in vivo function of CLAMT, we measured the endogenous levels of CLA and MeCLA in these mutants. We found that CLA highly accumulated in both mutants, whereas the MeCLA level was drastically reduced compared with WT (Fig. 3A), demonstrating that CLAMT serves as the main enzyme that catalyzes CLA methylation in vivo. However, considering the presence of detectable levels of MeCLA in the clamt mutants, another methyltransferase(s) may contribute to the CLA methylation step in vivo. Both clamt-1 and clamt-2 had increased axillary branching compared with No-0 WT (Fig. 3B). To evaluate whether that is due to reduced production of the SL branching hormone, we performed a shoot branching inhibition assay with these clamt mutants using a number of different SLs. Treatment with GR24 or MeCLA rescued the shoot branching phenotypes of both mutants, whereas CLA was not effective, confirming that CLA itself is not biologically active as a shoot branching inhibitor (Fig. 3C). In addition, these results illustrate a critical role of CLA methylation to produce the biologically active hormone(s) in Arabidopsis, supporting our in vitro experimental results using the receptor protein, AtD14 (10).

In order to compare the branching phenotype of the clamt mutant with other SL biosynthetic mutants, we generated the clamt-1 mutant in the Col-0 background by backcrossing with Col-0. After backcrossing seven times with Col-0, the phenotype of the clamt-1 (Col-0) was compared with the max1-4, max2-2, clamt-1, and max1-4 clamt-1 (Col-0) (SI Appendix, Figs. S5 and S6). These results suggest that MeCLA acts as a shoot branching inhibitor downstream of CLA methylation.
max3-11 (ccd7), and max4-8 (ccd8) mutants, all of which were in the Col-0 background. clamt-1 had fewer branches than the other SL biosynthetic mutants, but still significantly more than WT Col-0 (SI Appendix, Fig. S5 A and B). Notably, the clamt mutant exhibited a shoot branching phenotype similar to that of the lbo mutant (SI Appendix, Fig. S5 C and D). These results further support the idea that the methylation of CLA has an important role in generating the biologically active shoot branching inhibiting hormone.

Grafting Experiments Using the clamt Mutant. In previous reports, a series of grafting experiments using SL mutants revealed the relationship between the biosynthetic enzymes and the root-to-shoot translocation of the corresponding biosynthetic precursors. For example, the phenotype of the max4 mutant was restored by grafting onto the max1 mutant rootstock, suggesting that the substrate of MAX1 is able to translocate long distances from root to shoot (21). In addition, this result demonstrated that MAX1, a cytochrome P450, is functioning downstream of MAX4/CCD8—oxidizing CL, the product of CCD8—which was indeed experimentally verified later (10). Moreover, these results suggest that CL may be able to translocate from root to shoot, although the experimental evidence for this has not yet been provided.

We therefore grafted the max4 mutant shoot onto the max1 mutant root to evaluate the translocation of CL from root to shoot. We analyzed CL in the shoot (max4) part of the grafted plants, in which the branching phenotype was completely restored as was reported (SI Appendix, Fig. S6 A) (21). In this grafted plant, CL was successfully detected in the shoot extracts (Fig. 4A and SI Appendix, Fig. S6B). As we reported previously, the max1 mutant accumulated an extremely high level of CL. Thus, it would be possible that the translocation of CL occurs only when the max1 mutant is used as the rootstock. We therefore grafted the max4 mutant onto the WT rootstock, and even in this grafting combination CL was detected in the shoot part (Fig. 4A and SI Appendix, Fig. S6C), although the CL level in the shoot was lower than for the max4/max1 (shoot/root) grafting combination. These results provide direct evidence for the root-to-shoot translocation of the SL biosynthetic precursor, CL.

Next, in order to assess the translocation ability of the MAX1 product, CLA, we performed grafting experiments using the clamt-1 and the max1-4 mutants. Because the background of each mutant was different (No-0 for clamt-1, Col-0 for max1-4), we prepared a control plant; namely, the shoot of the max1-4 mutant was grafted onto the WT No-0 rootstock, which resulted in a favorable restoration of the max1-4 branching phenotype. In a subsequent experiment, the max1-4 phenotype was also restored by grafting onto the clamt-1 mutant (Fig. 4B and SI Appendix, Fig. S7A). This result suggests that CLA is transmissible from root to shoot, and it might be converted into a bioactive substance for shoot branching inhibition. These results also confirm that CLAMT functions downstream of MAX1. Next, to examine the translocation ability of the CLAMT product such as MeCLA, we performed grafting experiments using the clamt-1 mutant and WT plants. The branching phenotype of clamt-1 was not complemented by grafting onto a WT rootstock in both the Col-0 and the No-0 backgrounds, indicating that the products downstream of MeCLA.
CLAMT are not translocated from root to shoot (Fig. 4C and SI Appendix, Fig. S7 B–D). By contrast, the branching phenotype of the lbo scion was partially rescued by a WT rootstock as observed in a previous study (13), and more clearly by a d14 rootstock, in which SL biosynthesis is activated (SI Appendix, Fig. S8A). Furthermore, a WT rootstock moderately rescued the branching phenotype of the clamt lbo double mutant, which displayed an additive branching phenotype compared with the clamt and lbo single mutants (SI Appendix, Fig. S8 B and C), supporting the idea that the LBO product can move upward from the root. However, the insensitivity of the clamt scion to a WT rootstock is inconsistent with the hypothesis above. This point will be discussed below.

Expression of the CLAMT Gene. To analyze the expression pattern of CLAMT in Arabidopsis, we generated transgenic lines expressing CLAMTpro::GUS, in which β-glucuronidase (GUS) expresses under the regulation of the CLAMT promoter. CLAMTpro::GUS showed the strongest expression in the roots (Fig. 5A). This pattern correlated well with the higher levels of endogenous MeCLA in roots (Fig. 3A). CLAMTpro::GUS was also expressed in the vascular tissue, the flowers, and the basal part of cauline branches and the siliques (Fig. 5 B–E). Similar expression patterns have been reported for other SL biosynthetic genes although not entirely overlapping (13, 21, 22). The expression of CLAMTpro::GUS was particularly strong in the nodal part of young axillary buds, much more so than in

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**Fig. 3.** Analysis of the Arabidopsis clamt mutants in the No-0 background. (A) Quantitative analysis of endogenous CLA and MeCLA levels in Arabidopsis WT (No-0) and the clamt mutants. Data are the means ± SD (n = 4). (B) Shoot branching phenotype of Arabidopsis WT (No-0) and the clamt mutants. The number of axillary shoots (>5 mm) per plant of 44-d-old plants is shown as the mean ± SD (n = 9). Right panels show the pictures of the aboveground part of these plants. (C) Effect of SLs (10 μM) on axillary bud outgrowth of Arabidopsis. A solution (10 μL) containing each compound was applied to axillary buds every other day for 29 d. The number of axillary shoots (>5 mm) per 46-d-old plant is shown as the mean ± SD (n = 8). Different letters indicate significant differences at P < 0.05, Tukey’s honestly significant difference (HSD).
the older ones (SI Appendix, Fig. S9), suggesting that CLAMT functions locally to suppress bud outgrowth.

Previous reports have suggested the presence of a negative feedback regulation for some SL biosynthetic genes by endogenous or exogenously applied SL (1, 23). In order to assess the SL-dependent regulation of the CLAMT gene expression, we performed qRT-PCR analysis using whole seedlings or roots of 14-d-old WT and SL-related mutants, where MAX3 (24), MAX4 (22, 23), and CLAMTpro::GUS (Fig. 5A) are strongly expressed. CLAMT expression levels were not altered in any of the SL mutants, suggesting that CLAMT expression is not regulated by endogenous SL levels (SI Appendix, Fig. S10 A and B). This observation is supported by the fact that CLAMT is not included in SL-responsive genes in a recent transcriptome analysis (25). In contrast, the expression of MAX3 and MAX4 was slightly elevated in the clamt mutant compared with that in WT, but was weaker than that in other SL-related mutants in whole seedlings (SI Appendix, Fig. S10A). In roots, the up-regulation of MAX4 in the clamt mutant was significant, as observed in other SL-related mutants (SI Appendix, Fig. S10B). These results support the idea that CLAMT is involved in producing bioactive SLs that regulate the expression of upstream biosynthetic genes in Arabidopsis.

**Discussion**

We have previously identified an endogenous SL-like molecule in Arabidopsis (9), which was later shown to be MeCLA (10). Neither CL nor CLA physically interacts with the receptor protein, AtD14, while MeCLA does, strongly suggesting that the methylation of CLA is critical for converting a biologically inactive biosynthetic precursor into a bioactive substance in the shoot branching inhibition pathway in Arabidopsis. Here, we report the characterization of CLAMT that efficiently methylates CLA. The Arabidopsis mutants that are defective in CLAMT showed an increased level of CLA, while the level of MeCLA was significantly reduced. In addition, these clamt mutants exhibited increased branching phenotypes, demonstrating an essential role of CLAMT in producing biologically active molecules that inhibit shoot branching. On the other hand, MeCLA was still detectable in the clamt mutants, suggesting that there might be another methyltransferase(s) that can catalyze CLA methylation, although the contribution of such an enzyme(s) should be much smaller than that of CLAMT. Moreover, we found that the branching phenotype of the clamt mutant is weaker than that of other SL biosynthetic mutants, such as max1, max3, and max4. There are multiple possible reasons to explain this weaker branching phenotype of the clamt-1 mutant. First, the clamt-1 mutant is still producing detectable amounts of MeCLA as mentioned above, and this remaining MeCLA or its downstream metabolites may still weakly inhibit shoot branching in the clamt-1 mutant. Second, some SLs other than the products in the MeCLA pathway might inhibit shoot branching in the clamt-1 mutant. In fact, the presence of orobanchol and some other four ring-type SLs in Arabidopsis were reported (26), although we were unable to detect these. It is also important to clarify whether CLAMT is involved in the production of MeCLA derivatives (4-OH-MeCLA and 16-OH-MeCLA) that were recently identified in Arabidopsis (14).
MeCLA was reported to be a potential substrate for LBO. In agreement with this biochemical property, the Arabidopsis lbo mutant accumulated a larger amount of endogenous MeCLA. In addition, the lbo mutant was insensitive to exogenously applied MeCLA (13). These results suggest that the downstream product(s) of LBO, but not MeCLA, functions as the active hormone in the shoot branching inhibition pathway. Very recently, the reaction product of LBO when MeCLA was used as a substrate was identified to be 1'-OH-MeCLA (14). However, the main product was not 1'-OH-MeCLA, but the demethylation product, CLA. The authors proposed a possibility that 1'-OH-MeCLA is nonenzymatically converted into CLA because of its instability. It would also be possible, however, that LBO mainly catalyzes the oxidative demethylation of MeCLA and that 1'-OH-MeCLA is a reaction intermediate of the demethylation reaction. Moreover, another possibility is that 1'-OH-MeCLA is further converted into another metabolite by an unknown enzyme(s), and this metabolite may act as the true active hormone in shoot branching inhibition (14). In either case, our results clearly demonstrate that the CLA methylation step plays an essential role in synthesizing the active hormone for shoot branching inhibition. Because the clamt mutants can still synthesize a measurable level of MeCLA (Fig. 3A), the more severe phenotype observed in the clamt lbo mutant may be explained if both MeCLA (or its metabolite that does not pass the LBO pathway) and 1'-OH-MeCLA (or its unknown metabolite) independently function as bioactive hormones in inhibiting shoot branching as previously discussed to explain the weak phenotype of the lbo mutant (13). A clear understanding of the biochemical function of LBO will be necessary to elucidate the whole picture of SL biosynthesis, including the characterization of the as-yet-unidentified active hormone structure in the shoot branching inhibition pathway.

Grafting experiments using the clamt mutant demonstrated that CLA, but not MeCLA or its downstream metabolite(s), can be translocated long distance from root to shoot (Fig. 4 B and C). In contrast, the branching phenotype of the lbo mutant could be restored by grafting onto a WT rootstock, suggesting that the LBO downstream product(s) is transmissible (13) (SI Appendix, Fig. S8A). These results appear to be contradictory. However, it might be possible that MeCLA, which was produced from root-derived CL and CLA, accumulated and inhibited shoot branching in the scion of the lbo WT graft, but not in that of the clamt WT graft. Alternatively, CLAMT and LBO may function partially independently for producing shoot branching inhibiting hormones because both the clamt and lbo mutants are weak and additive (SI Appendix, Fig. S8B). Further experiments, such as local quantification of MeCLA in the grafted plants and substrate specificity analysis of CLAMT toward hydroxylated CLA derivatives (14), will provide clues to evaluate these possibilities.

Interestingly, possible CLAMT and LBO orthologous genes are widely distributed in seed plants, including rice, in which CLA was identified as an endogenous product (10), suggesting that rice also produces MeCLA-type SLs. This is supported by the detection of as-yet-unidentified SLs, tentatively coined methoxy-5-deoxystrigol isomers, in rice (27). Indeed, a phylogenetic analysis of SABATH genes shows that there are two rice genes in the same clade as the Arabidopsis CLAMT (28). Canonical SLs such as 4DO and orobanchol have been identified in rice. Thus, if MeCLA, and its downstream SLs, exist in rice, rice would be an appropriate species to investigate the functional differences between canonical and noncanonical SLs.

In conclusion, we have successfully identified and characterized a CLA methyltransferase in Arabidopsis. This will significantly contribute to understanding how structurally diverse SL molecules regulate plant growth and development and act as rhizosphere signals for symbiotic and parasitic interactions.

Materials and Methods

Plant Materials and Growth Conditions. We used Arabidopsis ecotype Col-0 and No-0 as the WT and max1-4 (9), max3-11 (1), max4-7 (1), and atd14-2 (9) mutants. The clamt mutants (RATM11-1866-1, clamt-1 and RATM53-2997-1, clamt-2) were obtained from the RIKEN BioResource Research Center. Genotyping was carried out by a PCR-based method using the primers listed in SI Appendix, Table S1. The clamt-1 and clamt-2 mutants were originally in the No-0 background. To compare their phenotypes with other SL biosynthetic mutants, all of which were in the Col-0 background, they were backcrossed with Col-0 seven times. The lbo (Col-0) mutant, which was generated by backcrossing lbo-1 (Wassilewskija) with Col-0 six times, was kindly provided by Christine Beveridge, The University of Queensland, St. Lucia, QLD, Australia. The clamt lbo mutant was generated by crossing clamt-1 (Col-0) and lbo (Col-0) mutants. Details of growth conditions and other experiments are described in SI Appendix, SI Materials and Methods.

Data Availability. All study data are included in the article and/or SI Appendix.

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