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# A carlactonoic acid methyltransferase that contributes to the inhibition of shoot branching in Arabidopsis

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**This PDF file includes:**

Main Text
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## Abstract

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 (At4g36470) efficiently catalyzes methyl esterification of CLA using *S*-adenosyl-$_L$-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 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 Statement

Strigolactones (SLs) are a group of apocarotenoid hormones that regulates 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.

## Main Text

### Introduction

The apocarotenoid strigolactones (SLs) act as plant hormones that regulate shoot branching (1, 2). They also have multiple hormonal roles in diverse developmental processes in plants (3-5). In addition, SLs serve as rhizosphere signals for symbiotic and parasitic interactions with arbuscular mycorrhizal fungi and root parasitic plants, respectively (6, 7). In 2012, a key biosynthetic intermediate called carlactone (CL) was identified through *in vitro* functional analysis of three enzymes, DWARF27 (D27), CAROTENOID CLEAVAGE DIOXYGENASE7 (CCD7), and CCD8 (8) (Fig. 1). Later, CL was identified as an endogenous metabolite both in Arabidopsis and rice. In addition, CL was converted to SLs such as 4-deoxyorobanchol (4DO) and orobanchol *in vivo* by feeding experiments using a stable isotope-labeled CL (9). A cytochrome P450, MORE AXILLARY GROWTH1 (MAX1), in Arabidopsis catalyzes a three-step oxidation of the C-19 of CL to yield a carboxylated analog, carlactonoic acid (CLA) (10) (Fig. 1), while one of the five MAX1 homologs in rice, Os01g0700900 (Os900), is a multi-functional enzyme catalyzing the conversion of CL into a four ring-type SL, 4DO (11). On the other hand, another MAX1 homolog, Os01g0701400 (Os1400) catalyzes hydroxylation of 4DO to produce orobanchol (11), showing that MAX1 homologs have functional diversity (Fig. 1).

Previously, we identified an endogenous SL-like compound in Arabidopsis, which we first named SL-LIKE1 (9). SL-LIKE1 was later demonstrated to be the methyl esterified analog of CLA, methyl carlactonoate (MeCLA) (10) (Fig. 1). SLs are perceived by an $\alpha/\beta$-fold hydrolase receptor, DWARF14 (D14), and they are hydrolytically degraded by D14 after signal transmission (12). Interestingly, we found that neither CL nor CLA could physically interact with Arabidopsis DWARF14 (AtD14), while MeCLA could (10). These results suggest that MeCLA is a biologically active hormone in the shoot branching inhibition pathway in Arabidopsis, and that the methylation of CLA is a critical step to convert a biologically inactive precursor to an active hormone molecule. Recently, a new SL biosynthetic enzyme, LATERAL BRANCHING OXIDOREDUCTASE (LBO), which belongs to the 2-oxoglutarate-dependent dioxygenase family, was identified in Arabidopsis (13). LBO catalyzes the formation of a MeCLA+16 Da reaction product from MeCLA. The Arabidopsis *lbo* knockout mutant exhibits a weaker shoot branching phenotype than the several SL biosynthetic mutants such as *max4* (defective in CCD8) and accumulates MeCLA. These results suggest that the pathway downstream of MeCLA has a more significant role than does MeCLA in the shoot branching inhibition in Arabidopsis. More recently, the LBO reaction product was identified to be hydroxymethyl carlactonoate (1'-OH-MeCLA), while LBO also catalyzed demethylation of MeCLA to produce CLA (14) (Fig. 1).

In order to further understand the role of the MeCLA pathway in Arabidopsis, the identification of the elusive CLA methyltransferase is indispensable. Among the methyltransferase protein families, the SABATH family has been demonstrated to include enzymes that methylate phytohormones, such as gibberellin (15), jasmonate (16), auxin (17), and salicylic acid (18). Thus, we hypothesized that CLA methyltransferase(s) also belongs to the SABATH protein family. Among 24 SABATH members in Arabidopsis, the gene product of *At4g36470*, which we named CLA methyltransferase (CLAMT), indeed efficiently catalyzed CLA methylation. Our finding is consistent with a recent report that At4g36470 catalyzed the methylation of (11*R*)-CLA (19). In addition, the *clamt* knockout mutant highly accumulated CLA, whereas MeCLA was significantly reduced in the mutant. Notably, the *clamt* mutant exhibited an increased shoot branching phenotype. Taken together, we successfully identified a CLA methyltransferase in Arabidopsis,

which has a critical role in producing biologically active substances in the shoot branching regulation pathway.

## Results

**Biochemical screening of CLAMT in the Arabidopsis SABATH protein family.** Because of their role in methylation of plant hormones (15-18), we hypothesized that CLA methyltransferase(s) is a SABATH protein family member. The Arabidopsis genome contains 24 genes belonging to the *SABATH* family. We examined the 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 (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, 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 co-expressing At4g36470 and the CLA biosynthetic enzymes (AtD27, MAX3/CCD7, MAX4/CCD8, and MAX1) (Fig.S3). Phylogenetic analysis of all 24 SABATH family proteins in Arabidopsis suggests that there are no closely related homologs of *At4g36470* (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 RIKEN Arabidopsis transposon-tagged collection in the Nossen (No-0) background (line RATM11-1868-1; *clamt-1* and RATM53-2997-1; *clamt-2*) (Fig. S4A) (20). In both of these alleles, the *CLAMT* transcript levels were significantly reduced compared with wild-type (WT) No-0 (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, other 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 if 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*, *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 (Figs. S5A, B). Notably, the *clamt* mutant exhibited a shoot branching phenotype similar to that of the *lbo* mutant (Figs. S5C, 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 (Fig. S6A) (21). In this grafted plant, CL was successfully detected in the shoot extracts (Fig. 4A, 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, 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, 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 CLAMT are not translocated from root to shoot (Fig. 4C, Figs. S7B-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 (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 (Figs. S8B, 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 (Figs. 5B-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 the older ones (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-day-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 (Figs. S10A, B). This observation is supported by 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 (Fig. S10A). In roots, the up-regulation of *MAX4* in the *clamt* mutant was significant as observed in other SL-related mutants (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 other methyltransferase(s) that can catalyze CLA methylation, although the contribution of such 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, function 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 (Figs. 4B 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(are) transmissible (13) (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 (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 endogenous products (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 non-canonical 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 (RATM11-1868-1, *clamt-1*; RATM53-2997-1, *clamt-2*) were obtained from RIKEN BioResource Research Center. Genotyping was carried out by a PCR-based method using the primers listed in 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 Dr. Christine Beveridge. 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 Materials and Methods*.

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## Figures legends

**Figure 1.** Proposed biosynthetic pathway of SLs. Blue, red, green, and orange letters indicate genes of rice, Arabidopsis, pea, and petunia, respectively. 2OGD, 2-oxoglutarate-dependent dioxygenase; CCD, carotenoid cleavage dioxygenase.

**Figure 2.** Conversion of CLA to MeCLA by CLAMT. Enzymatic reaction was performed using CLA as a substrate in the presence of SAM. The reaction product of the cell lysate expressing the empty vector or At4g36470 (CLAMT) was analyzed by LC-MS/MS (Sciex X500R QTOF). The MS/MS chromatogram for MeCLA (*m*/*z* 347.18 > 97.03) (left) and MS/MS scan spectra of fragment ions between *m*/*z* 50 to 380 from a precursor ion with *m*/*z* 347.18 (right) are shown.

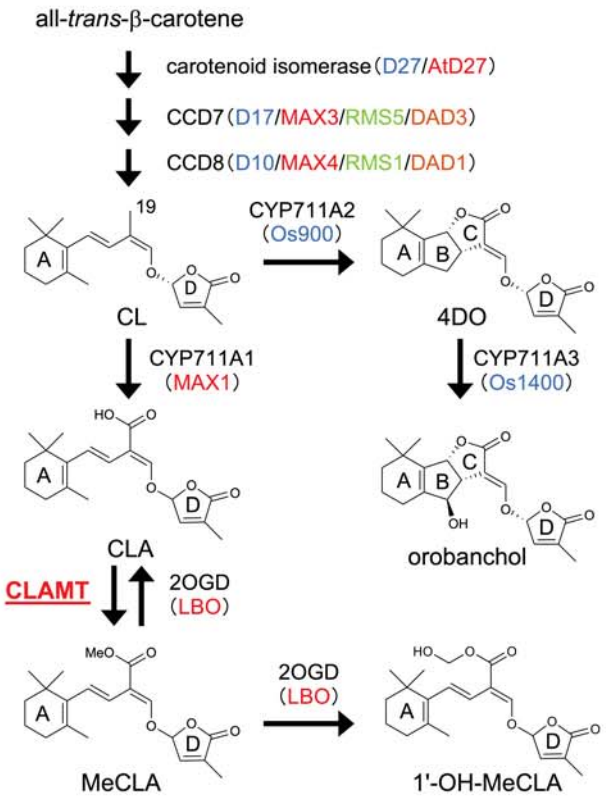
**Figure 3.** Analysis of the Arabidopsis *clamt* mutants in the No-0 background. (A) Quantitative analysis of endogenous CLA and MeCLA levels in Arabidopsis WT (No-0) and the *clamt* mutants.

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-day-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 days. The number of axillary shoots (> 5 mm) per 46-day-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).

**Figure 4.** Analysis of the root to shoot translocation of endogenous SL biosynthetic intermediates in the grafted plants. (A) Quantitative analysis of endogenous levels of CL in the shoot of the grafted plants. Data are the means ± SD (*n* = 3). (B) Shoot branching phenotype of 63-day-old grafted plants using WT (No-0 or Col-0), the *max1-4* (Col-0), and the *clamt-1* (No-0) mutants. The number of axillary shoots (> 5 mm) per plant is shown as the mean ± SD (*n* = 10). (C) Shoot branching phenotype of 77-day-old grafted plants using WT (No-0) and *clamt-1* (No-0) mutants. The number of axillary shoots (> 5 mm) per plant is shown as the mean ± SD (*n* = 12). Different letters indicate significant differences at $P < 0.05$, Tukey's HSD.

**Figure 5.** The *GUS* expression patterns in transgenic lines carrying *CLAMTpro*::*GUS*. The 14-day-old (A, B) and 40-day-old (C-E) plants were stained with an X-gluc-containing solution. A, root; B, aerial tissue; C, basal part of the cauline branch; D, flower; E, silique. GUS staining was performed for 1 h (A), 3 h (B), and 20 h (C-E). The bar indicates 1 mm. LR, lateral root; PR, primary root; CB, cauline branch; CL, cauline leaf; PS, primary shoot.

# Fig. 1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CCD8 (D10/MAX4/RMS1/DAD1)
19
CL
CYP711A2
(Os900)
4DO
CYP711A1
(MAX1)
CYP711A3
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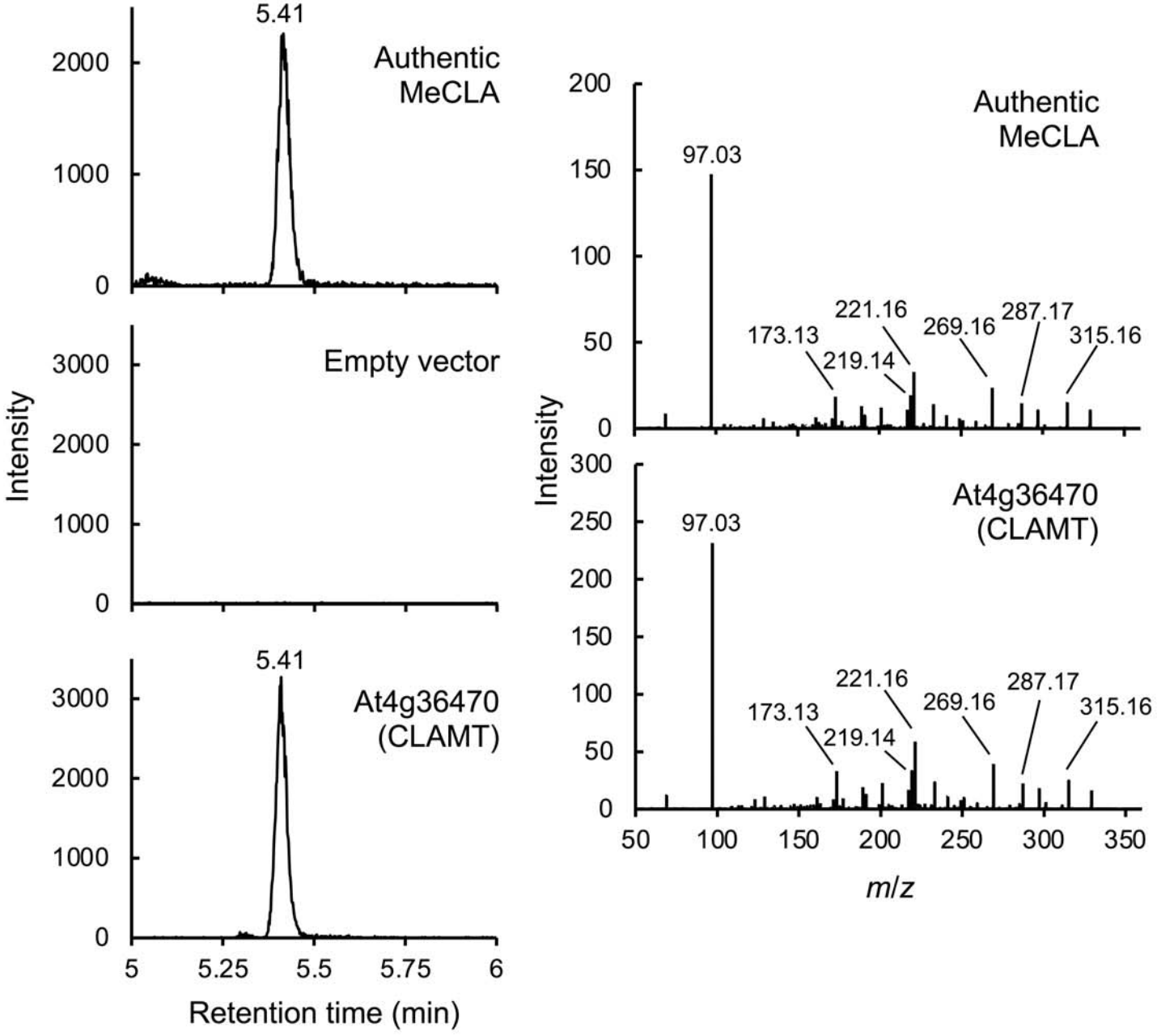
MeCLA
2OGD
(LBO)
1'-OH-MeCLA

# Fig. 2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Empty vector
At4g36470 (CLAMT)
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Retention time (min)
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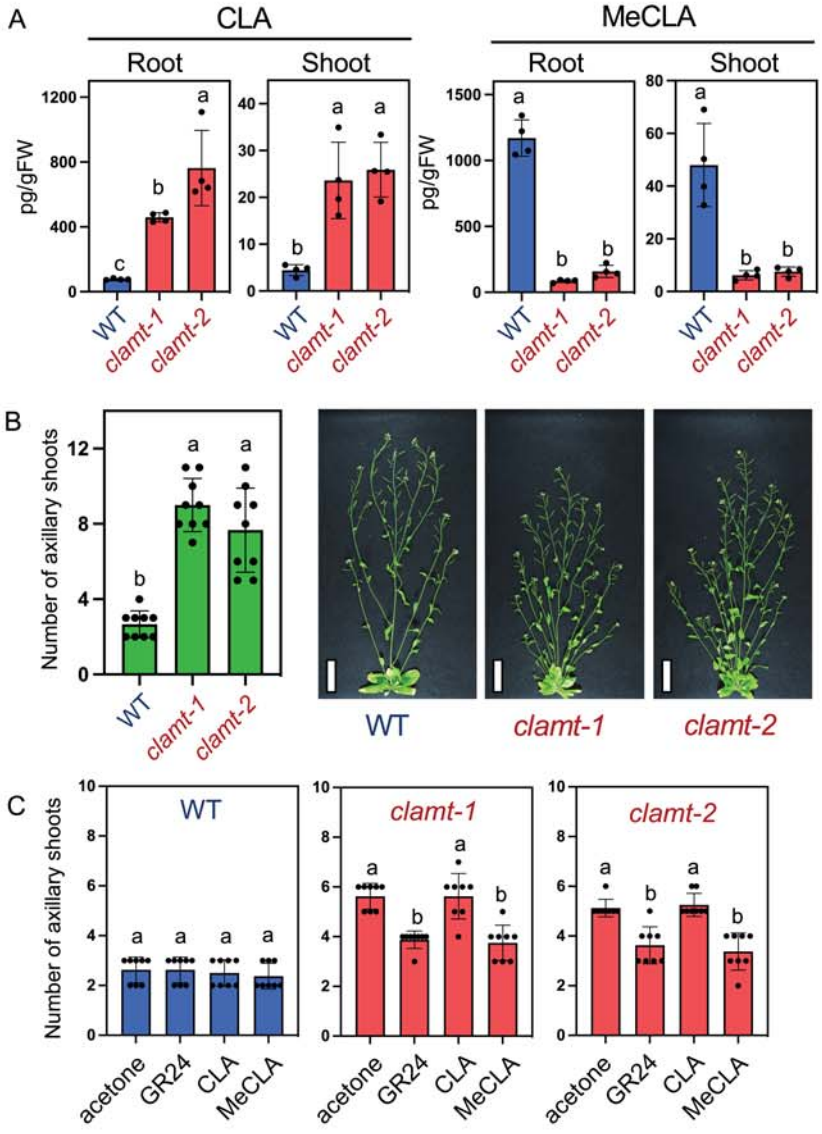
269.16
287.17
315.16
m/z

# Fig. 3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CLA
MeCLA
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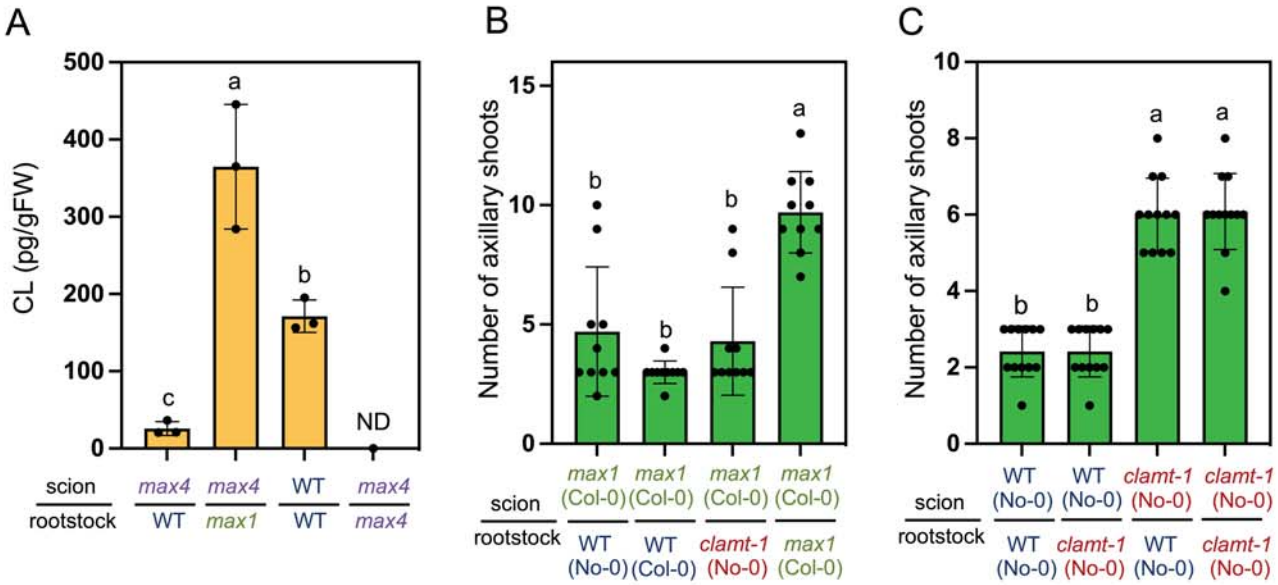
Shoot
pg/gFW
WT
clamt-1
clamt-2
B
Number of axillary shoots
C
WT
clamt-1
clamt-2
acetone
GR24
CLA
MeCLA

# Fig. 4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400
300
200
100
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a
b
c
ND
scion
rootstock
max4
WT
max4
max1
WT
WT
max4
max4
B
Number of axillary shoots
15
10
5
0
b
b
b
a
max1 (Col-0)
max1 (Col-0)
max1 (Col-0)
max1 (Col-0)
scion
rootstock
WT (No-0)
WT (Col-0)
clamt-1 (No-0)
max1 (Col-0)
C
Number of axillary shoots
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8
6
4
2
0
b
b
a
a
WT (No-0)
WT (No-0)
clamt-1 (No-0)
clamt-1 (No-0)
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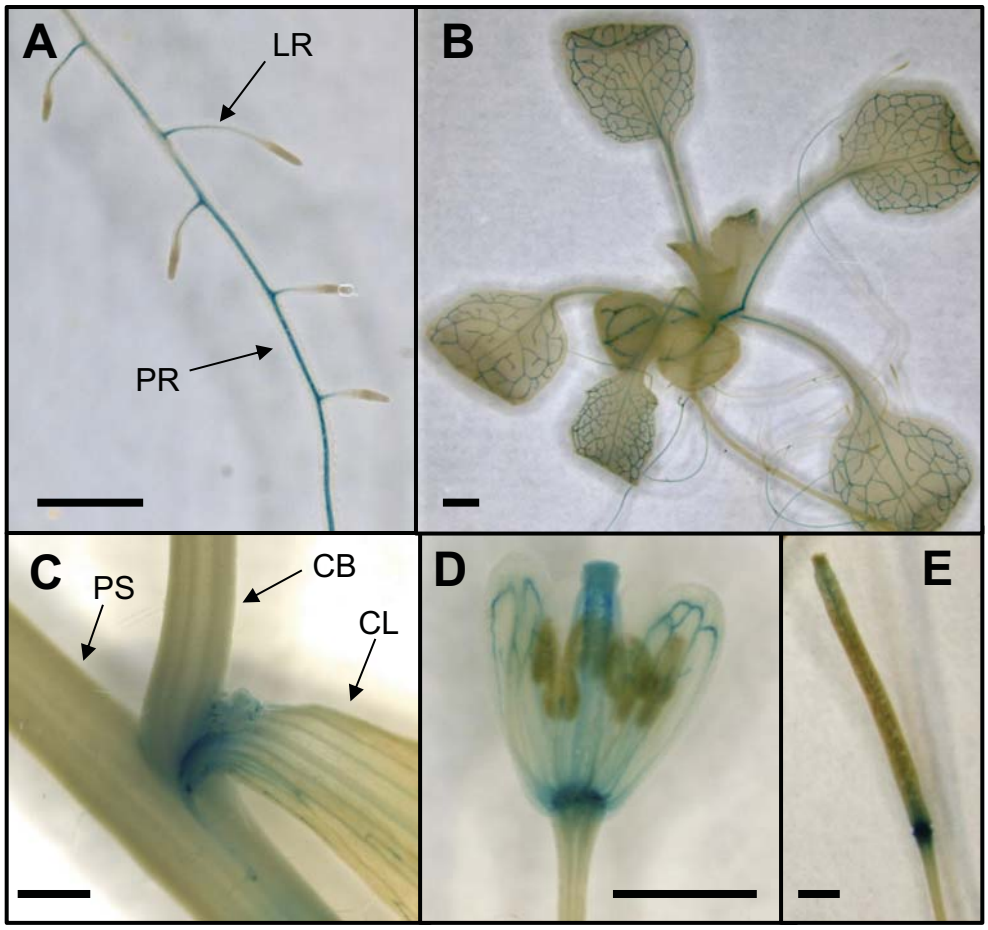
WT (No-0)
clamt-1 (No-0)

# Fig. 5



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## Supplementary Information for

# A carlactonoic acid methyltransferase that contributes to the inhibition of shoot branching in Arabidopsis

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**This PDF file includes:**

## SI Materials and Methods

**Chemicals.** GR24 (racemic mixture) was purchased from Chiralix. Preparations of CLA, MeCLA, and their stable isotope-labeled compounds were described in our previous reports (1).

**Expression of CLAMT in *E. coli*.** The coding sequence of the cDNA for CLAMT (At4g36470) was amplified by PCR using the primers listed in Table S1. The PCR product was digested by BamHI and SalI and cloned into the same sites of pET47b (Novagen) to yield CLAMT-pET47b. *E.coli* BL21 star (DE3) was used for the expression. Overnight growing culture (1 mL) was inoculated to a fresh LB medium (100 mL) containing 50 μg/mL kanamycin. After $OD_{600}$ reached 0.6-0.8, IPTG (0.1 mM) was added and further incubated at 16°C for 16 h. The cells were collected by centrifugation and then suspended and sonicated in the lysis buffer (50 mM Tris-HCl (pH8.0) containing 500 mM NaCl, 20 mM imidazole, 1 mM DTT, 10% Glycerol, and 1% Tween 20). The supernatant from the resulting cell lysate was used as the crude enzyme extract.

**Enzyme assay of CLAMT**. The CLA methyltransferase activity test was carried out at 37°C in 150 μL of a reaction buffer containing the crude enzyme extract [7.5 μg (CLAMT) or 15 μg (empty vector) of total protein], 25 pmol of CLA, 2 mM SAM in PBS buffer (pH 7.4). After 2 h, the enzyme reaction mixture was extracted with 300 μL of ethyl acetate (EtOAc), and the organic phase was evaporated to dryness under nitrogen gas. Each sample was dissolved with acetonitrile and subjected to LC-MS/MS. LC-MS/MS analysis of MeCLA was carried out using an X500R QTOF (AB SCIEX) equipped with a CORTECS UPLC C18+ column (ϕ2.1×100 mm) (Waters). The elution of the samples was carried out with 0.05% (vol/vol) acetic acid/water (solvent A) and 0.05% (vol/vol) acetic acid/acetonitrile (solvent B) at a flow rate of 0.3 mL $min^{-1}$. The temperature of the column was set to 35°C. LC conditions were as follows: 30% solvent B (0–0.5 min), a gradient from 30% to 75% solvent B (0.5–2.5 min) and 75% to 95% solvent B (2.5–6.0 min). The MS/MS analysis was operated under the positive ion mode as following parameters: ionspray voltage, 5500 V; declustering potential, 20 V; collision energy, 20 V; curtain gas, 40 psi; GS1, 30 psi; GS2, 40 psi; temperature, 450°C

**Transient expression analysis of CLAMT in *Nicotiana benthamiana*.** The full-length *CLAMT* was amplified from *Arabidopsis* Col-0 root cDNA using primers listed in Table S1. The amplified cDNA was then introduced into pIV1A_2.1 (2), and three individual colonies were confirmed by sequencing. The cDNA was subsequently introduced into the pBin-Plus binary vector (2), using the Gateway LR clonase II enzyme mix (Invitrogen). The binary vector was transformed into *A. tumefaciens* AGL0 by electroporation.

*N. benthamiana* plants were grown in individual pots with potting compost, placed in a greenhouse at 20°C and 65% relative humidity for 4 weeks and then used for infiltration with *A. tumefaciens* AGL0 as previously described (2). For the different gene combinations, equal concentrations of the *A. tumefaciens* strains harboring different constructs (grown to an $OD_{600}$ = 0.5) were mixed, and strains carrying empty vectors were used to compensate for the number of genes, if needed. In all combinations, an *A. tumefaciens* strain harboring a gene encoding the TBSV P19 protein was added to maximize protein production by suppression of gene silencing. The bacterial suspension was injected into the abaxial side of the leaf by using a 1 mL syringe without the needle. After 6 days, the infiltrated leaves were harvested and frozen in liquid nitrogen and stored at -80°C until further analysis. Seven biological replicates were used for each gene combination.

For SL analysis, 200 mg fine-ground agro-infiltrated *N. benthamiana* leaf tissue was used. EtOAc was used as the extraction solvent. The extraction followed a previously published method (3), except that GR24 was used as an internal standard. Strata® SI-1 Silica (55 μm, 70 Å, 200 mg/3 mL) columns were used for extract purification. The columns were preconditioned with 2 mL of EtOAc followed by 4 mL of hexane. Crude extract of *N. benthamiana* leaves in a mixture of EtOAc (50 μL) and 4 mL *n*-hexane was applied to a preconditioned silica column. The column was then washed with 2 mL absolute n-hexane. Subsequently, 3 mL of 10:90 hexane: EtOAc was used to elute SLs from the column. The eluent was dried under vacuum, reconstituted in 100 μL

acetonitrile/water (1:4), and filtered with a Micro Spin (centrifuge) filter 0.2NY (Thermo) before analysis by MRM LC-MS/MS. In UHPLC-MS/MS analysis, retention times and mass transitions were compared with authentic SL standards as previously described (2).

**Shoot branching inhibition assays.** For shoot branch inhibition assay, Arabidopsis seeds were sterilized in 2% PLANT PRESERVATIVE MIXTURE (PPM) solution (Plant Cell Technology) for 2 days at 4°C. The seeds were rinsed with water, and then placed on the half-strength Murashige and Skoog (MS) medium (4) containing 1% sucrose and 1% agar (pH 5.7) at 22°C under white fluorescence light (35 μmol $m^{-2}s^{-1}$) with a 16 h light/8 h dark photoperiod for 14 days. The seedlings were then transferred on the soil and further grown under the same condition. From the 4th day after the transfer, the application of SLs was repeated every other day. Each SL was dissolved in 0.1% Tween20 and 0.5% acetone solution and, 10 μL of the solution was applied directly to axillary buds.

**LC-MS/MS analysis of endogenous CL in Arabidopsis.** Analysis of endogenous CL was performed as previously described (5).

**LC-MS/MS analysis of endogenous CLA and MeCLA in Arabidopsis.** Seeds were sterilized in PPM solution for 3 days at 4°C, rinsed with water, and then placed on the half-strength MS medium containing 1% sucrose and 1% agar (pH 5.7) at 22°C under fluorescence white light (50-65 μmol $m^{-2}s^{-1}$) with a 16 h light/8 h dark photoperiod for 14 days. Plants were then transferred to a glass pot containing 400 mL of hydroponic solution (6) and grown under the same environmental condition for an additional 14 days. Hydroponic culture media were renewed after 7 days.

To analyze endogenous CLA and MeCLA, the roots or shoots samples (7 plants for a sample) were homogenized in 10 mL of acetone containing [1-$^{13}CH_3$]-*rac*-CLA and [10-$^{2}H_1$]-*rac*-MeCLA as internal standards. The filtrates were evaporated to dryness under nitrogen gas, dissolved in 3 mL distilled water and 1 mL saturated NaCl, and extracted with 5 mL of EtOAc twice. The combined EtOAc layer was divided into two samples, one is for CLA analysis, and the other one is for MeCLA analysis. For CLA analysis, the EtOAc fraction was evaporated to dryness under nitrogen gas, dissolved in 600 μL of EtOAc:*n*-hexane (5:95) containing 0.5% acetic acid, and loaded onto the Sep-Pak silica 1 cc vac cartridge (Waters). The columns were washed with 2 mL of the same solvent, and then eluted with 2 mL of EtOAc:*n*-hexane (30:70) containing 0.5% acetic acid. The eluates were evaporated to dryness under nitrogen gas, dissolved with 1 mL of acetonitrile and loaded onto the Bond Elut DEA (100 mg) 1 mL cartridge (Agilent). The columns were washed with 1 mL of acetonitrile, and then eluted with 1 mL of acetonitrile containing 1% acetic acid. The eluates were evaporated to dryness under nitrogen gas, dissolved with acetonitrile, and subjected to LC-MS/MS analysis.

For MeCLA analysis, the EtOAc fraction was evaporated to dryness under nitrogen gas, dissolved in 600 μL of *n*-hexane, and loaded onto the Sep-Pak silica 1 cc vac cartridge (Waters). The columns were washed with 2 mL of EtOAc:*n*-hexane (5:95), and then eluted with 2 mL of EtOAc:*n*-hexane (10:90) . The eluates were evaporated to dryness under nitrogen gas, dissolved with 600 μL of *n*-hexane and loaded onto the Sep-Pak cyanopropyl 1 cc vac cartridge (Waters). The columns were washed with 0.75 mL of *n*-hexane, and then eluted with 3 mL of *n*-hexane. The eluates were evaporated to dryness under nitrogen gas, dissolved with acetonitrile, and subjected to LC-MS/MS analysis.

LC-MS/MS analysis of CLA and MeCLA was carried out using a system consisting of a quadrupole/time-of-flight tandem mass spectrometer (TripleTof 5600, AB SCIEX) and an ultra high performance liquid chromatography (Nexera, Shimadzu) equipped with a reverse phase column (Acquity UPLC BEH-C18, ϕ1.0×100 mm, 1.7 μm; Waters).

For CLA analysis, the elution of the samples was carried out with 0.05% acetic acid (solvent A) and acetonitrile with 0.05% acetic acid (solvent B), and the mobile phase was changed from 30% B to 55% and 75% at 5 and 7 min after the injection, respectively, at a flow rate of 0.1 mL $min^{-1}$. MS/MS analysis conditions were as follows: Negative ion mode; Declustering potential, −75 V; collision energy, −16 V; and parent ion (*m/z*), 331.1 for unlabeled CLA and 332.2 for

labeled CLA. Quantification was carried out by using the fragment ion, *m/z* 113.025 for both unlabeled and labeled CLA.

For MeCLA analysis, the elution of the samples was carried out with solvent A and solvent B, and the mobile phase was changed from 30% B to 85% at 7 min after the injection, at a flow rate of 0.1 ml $min^{-1}$. MS/MS analysis conditions were as follows: Positive ion mode; Declustering potential, 55 V; collision energy, 28 V; and parent ion (*m/z*), 347.2 for unlabeled MeCLA and 348.2 for labeled MeCLA. Quantification was carried out by using the fragment ion, *m/z* 97.03 for both unlabeled and labeled MeCLA.

**Grafting experiments using Arabidopsis.** Grafting experiments were carried out according to a previous report (7). Seeds were sterilized and stratified by PPM for 2 days at 4°C. After being rinsed with sterile water, the seeds were put on the wetted membrane filters, and after 3-7 days, the hypocotyl parts were cut, and a shoot part was grafted with a root part of another plant. Six to seven days after grafting, grafted plants without adventitious roots were transferred on the soil, and further grown at 22°C under fluorescence white light (50-65 μmol $m^{-2}s^{-1}$) with a 16 h light/8 h dark photoperiod.

**Promoter-GUS analysis of CLAMT.** The 2122 bp upstream of the start codon of *CLAMT* was amplified from Arabidopsis genomic DNA by using primers, CLAMTpro-F-attB1 and CLAMTpro-R-attB2 (Table S1), and cloned into the pDONR207 vector (Invitrogen) by BP clonase (Invitrogen). After verified by DNA sequencing, the CLAMT promoter was introduced into the pGWB433 binary vector (8) by LR clonase II (Invitrogen) to generate a CLAMT promoter-fused *GUS* gene (*CLAMTpro*::*GUS*). Plant transformation, selection of transformants, and histochemical staining were mostly followed by the previous report (9). Briefly, the homozygous transgenic $T_3$ lines were soaked in the GUS stain solution [78 mM Phosphate buffer (pH 7.0), 2 mM $K_3[Fe(CN)_6]$, 2 mM $K_4[Fe(CN)_6]$, 0.1% TritonX-100, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc)] and incubated at 37°C in the dark. Then, tissues were washed twice with phosphate buffer (pH 7.0), and decolorized with 70% ethanol. We confirmed that independent 6 $T_3$ lines showed mostly the same expression patterns.

**qRT-PCR analysis.** Total RNA was isolated and purified using RNeasy Plant Mini Kit (Qiagen) from 14-day-old whole seedlings (3 seedlings per sample) (Fig. S4B and S10A) or Total RNA Extraction Kit Mini (Plant) (RBC Bioscience) from roots of 14-day-old seedlings (3-7 seedlings per sample) (Fig. S10B). ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) was used for cDNA synthesis from total RNA. The Mx3000P system (Agilent) was used to perform qRT-PCR by using the THUNDERBIRD Probe qPCR Mix (TOYOBO) and TaqMan probes (Fig. S4B and S10A) or the KOD SYBR qPCR mix (TOYOBO) (Fig. S10B). The primers and TaqMan probes are described in Table S1.

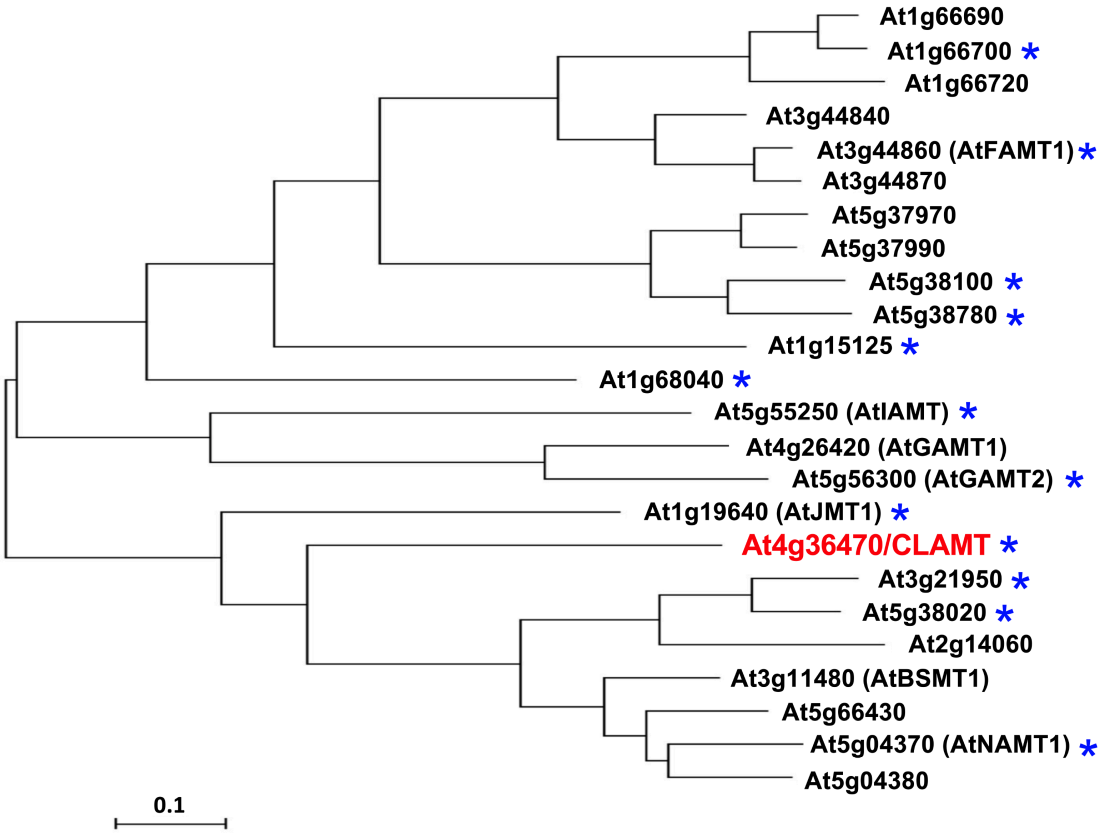


**Fig. S1**. Phylogenetic analysis of the Arabidopsis 24 SABATH family proteins. CLAMT (At4g36470) is shown in red. AtGAMT1 and AtGAMT2, gibberellin methyltransferases (10); AtIAMT, indole-3-acetic acid (IAA) methyltransferase (11); AtJMT1, jasmonic acid methyltransferase (12); AtBSMT1, salicylic acid methyltransferase (13); AtFAMT1, farnesoic acid methyltransferase (14); AtNAMT1, nicotinic acid methyltransferase (15). Blue asterisks indicate 13 SABATH proteins analyzed in this study.

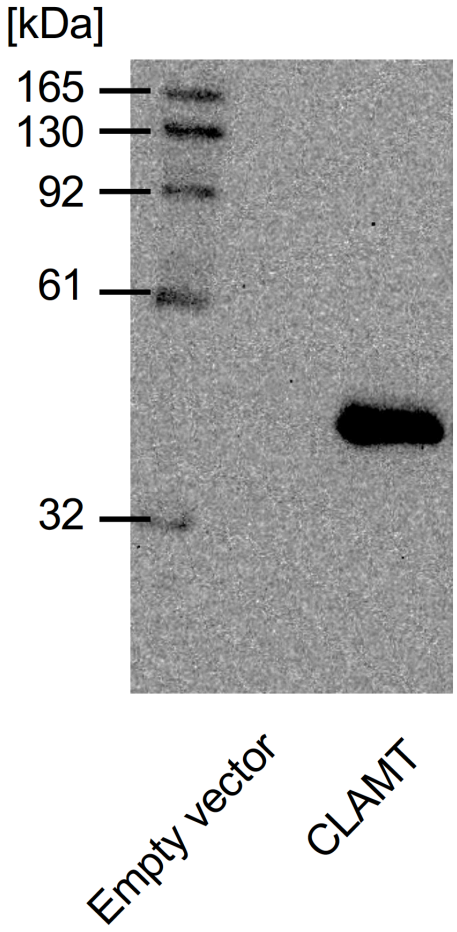


**Fig. S2**. Expression of the recombinant CLAMT protein in *E.coli*. The crude enzyme extract (36 μg of total protein) was separated by SDS-PAGE and analyzed by western blot by using anti-6×His monoclonal antibody 9C11 (FUJIFILM Wako). SuperSignal West Pico Chemiluminescent Substrate (Thermo) was used for the detection of His-tagged CLAMT. The crude enzyme extract from *E.coli* expressing the empty vector was used as a negative control.

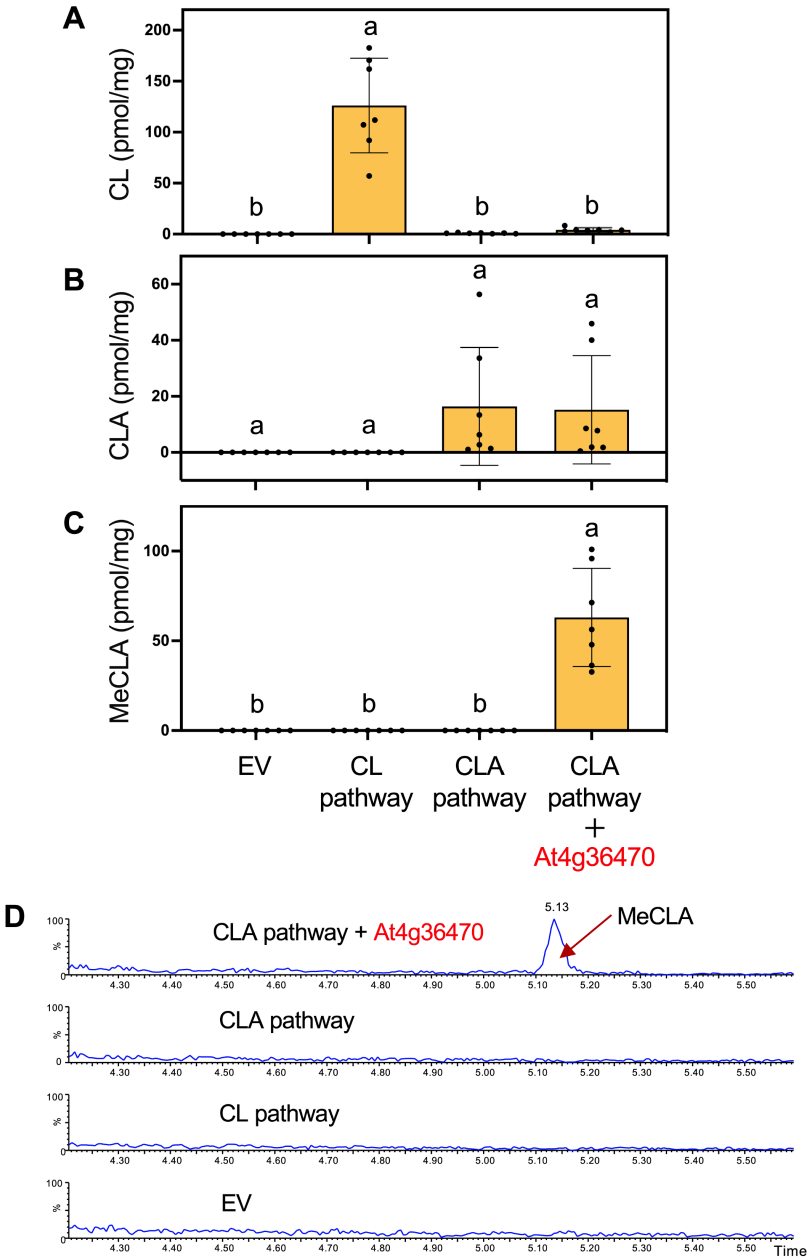


**Fig. S3**. Biochemical characterization of CLAMT by transient expression in *N. benthamiana*. The levels of CL (A), CLA (B), and MeCLA (C) in *N. benthamiana* leaves and the chromatograms of MeCLA (D) are shown. Expression of the CL pathway (*AtD27+MAX3+MAX4*) resulted in the production of about 120-130 pmol/mg of CL (A). Expression of the CLA pathway (*AtD27+MAX3+MAX4+MAX1*) resulted in the complete disappearance of CL (A), and the appearance of CLA (B). The discrepancy between the amount of CL disappearing (120-130 pmol) and the amount of CLA appearing (about 15-20 pmol) is caused by conjugation of CLA to (di-)hexoses by endogenous glycosyltransferase activity in *N. benthamiana*, as shown before (16). However, At4g36470/CLAMT successfully competed with this glycosyltransferase activity: when *At4g36470* was co-expressed with the CLA pathway, MeCLA was produced at about 60-70 pmol/mg (C, D). Production of MeCLA was not observed in the *N. benthamiana* leaves expressing EV, CL, and CLA gene combinations (C, D). LC-MS/MS (Agilent 6460 LC-QQQ) was used for analysis and MRM transitions were as follows: CL, *m/z* 303>97; CLA, *m/z* 331>69; MeCLA, *m/z* 347>97. EV, empty vector. Data are the means ± SD ($n$ = 7). Different letters indicate significant differences at $P < 0.05$, Tukey's HSD.

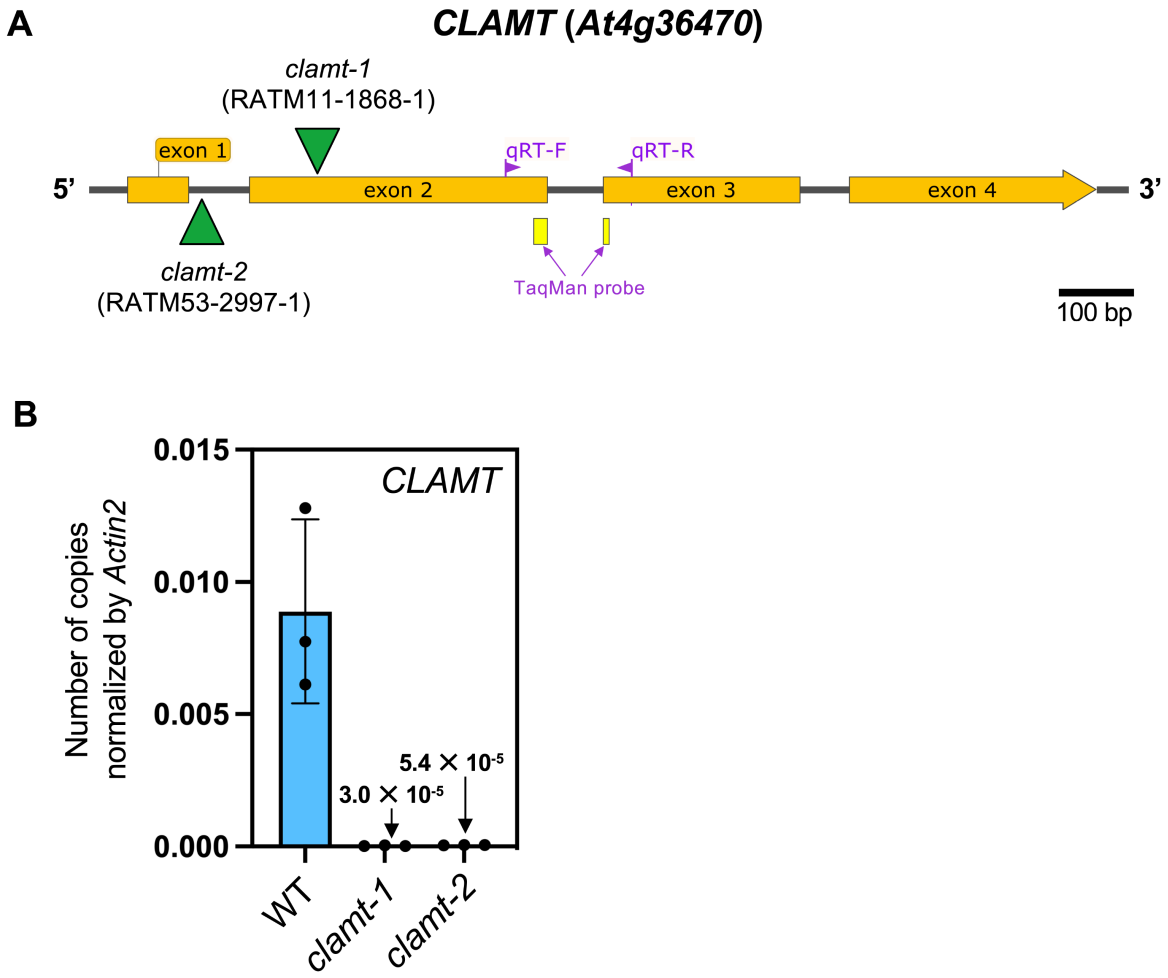


**Fig. S4**. Characterization of the Arabidopsis *clamt* mutants. A: Schematic diagram showing the *clamt* mutations in *CLAMT* (*At4g36470*). B: The transcript levels of *CLAMT* in the two independent alleles of the *clamt* mutant. Data are the means ± SD (*n* = 3).

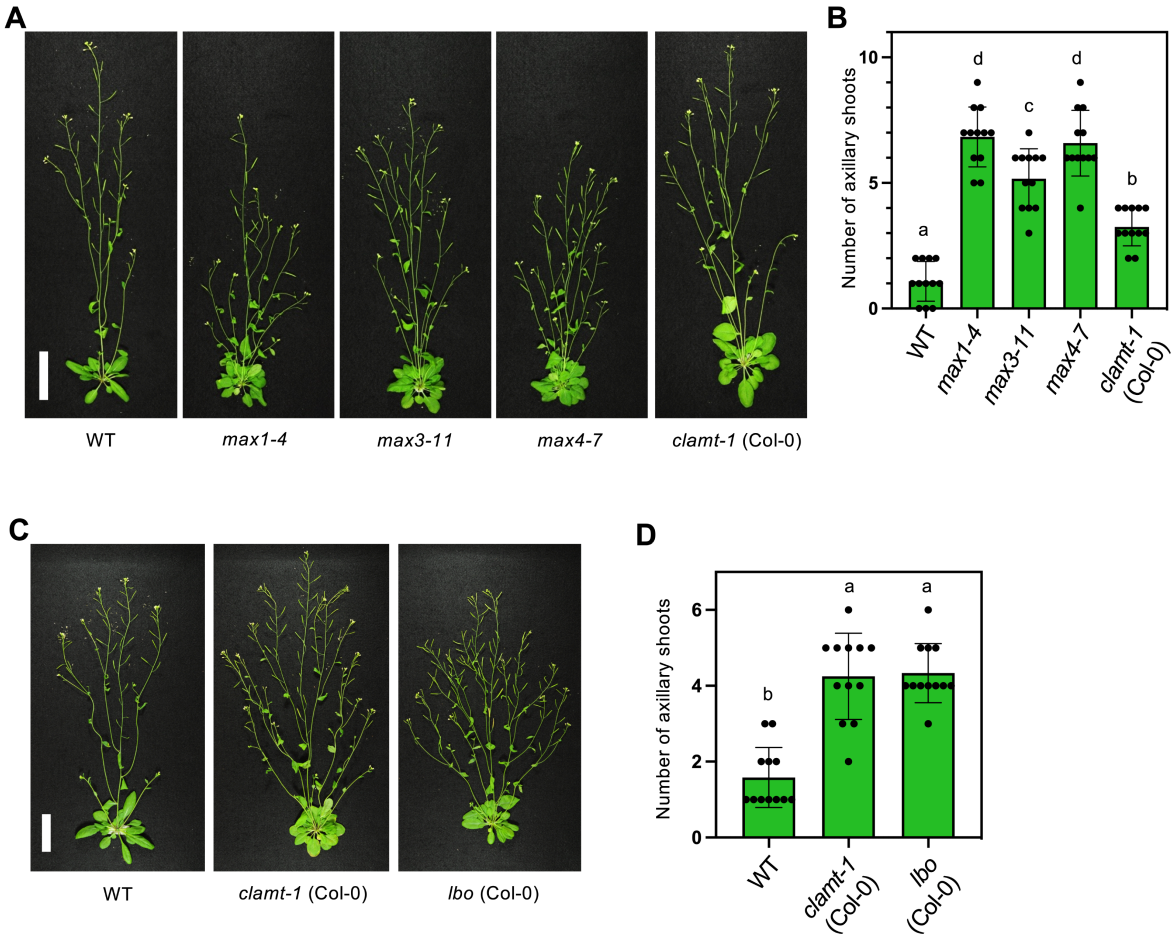


**Fig. S5**. Shoot branching phenotypes of the *clamt-1* mutant in the Col-0 background. (A, C) The aboveground parts of 39-day-old plants are shown. Scale bars = 5 cm. (B, D) The number of axillary shoots (> 5 mm) per plant is shown as the mean ± SD ($n$ = 12). Different letters indicate significant differences at $P < 0.05$, Tukey's HSD.

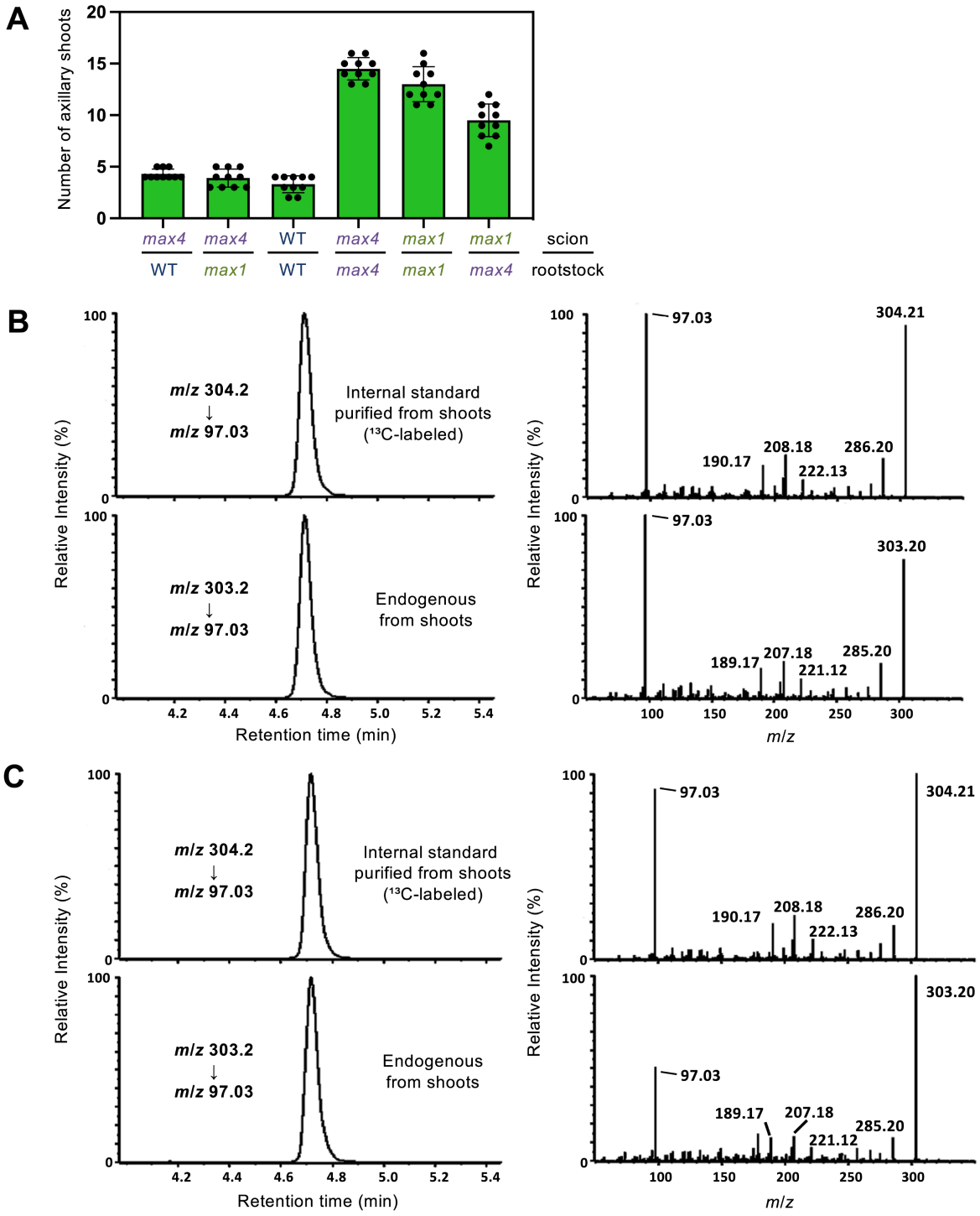


**Fig. S6**. Analysis of the root to shoot translocation of endogenous CL in the grafted plants. A: The number of axillary shoots (> 5 mm) of the grafted plant with each combination. Data are the means ± SD (*n* = 10). B and C: LC-MS/MS analysis of endogenous CL in the shoot of the grafted plants [the shoot/root combination is *max4*/*max1* in (B) and *max4*/WT in (C)]. Quantitative values of endogenous levels of CL in the shoot of the grafted plants are shown in Fig. 4A.

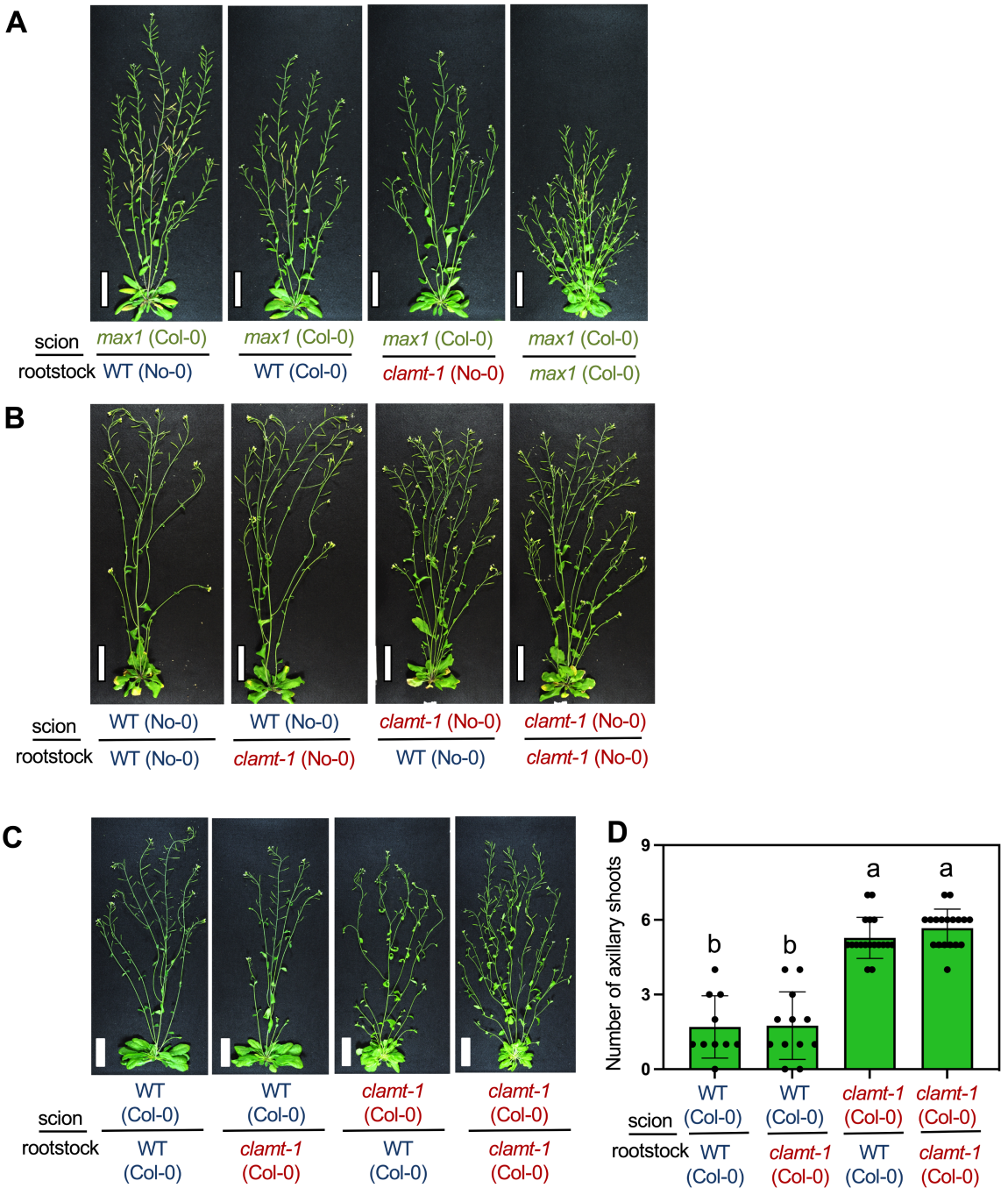


**Fig. S7**. Grafting experiments using the *clamt-1* mutant. (A) The aboveground parts of the plant presented in Fig. 4A are shown. (B) The aboveground parts of the plant presented in Fig. 4B are shown. Scale bars = 5 cm. (C, D) The aboveground parts and the number of axillary shoots of 70-day-old grafted plants using WT and the *clamt* mutant in the Col-0 background. In this experiment, the *clamt-1* mutant after backcrossing with Col-0 WT four times was used. The number of axillary shoots (> 5 mm) per plant is shown as the mean ± SD (*n* = 10-18). Different letters indicate significant differences at $P < 0.05$, Tukey's HSD. Scale bars = 5 cm.

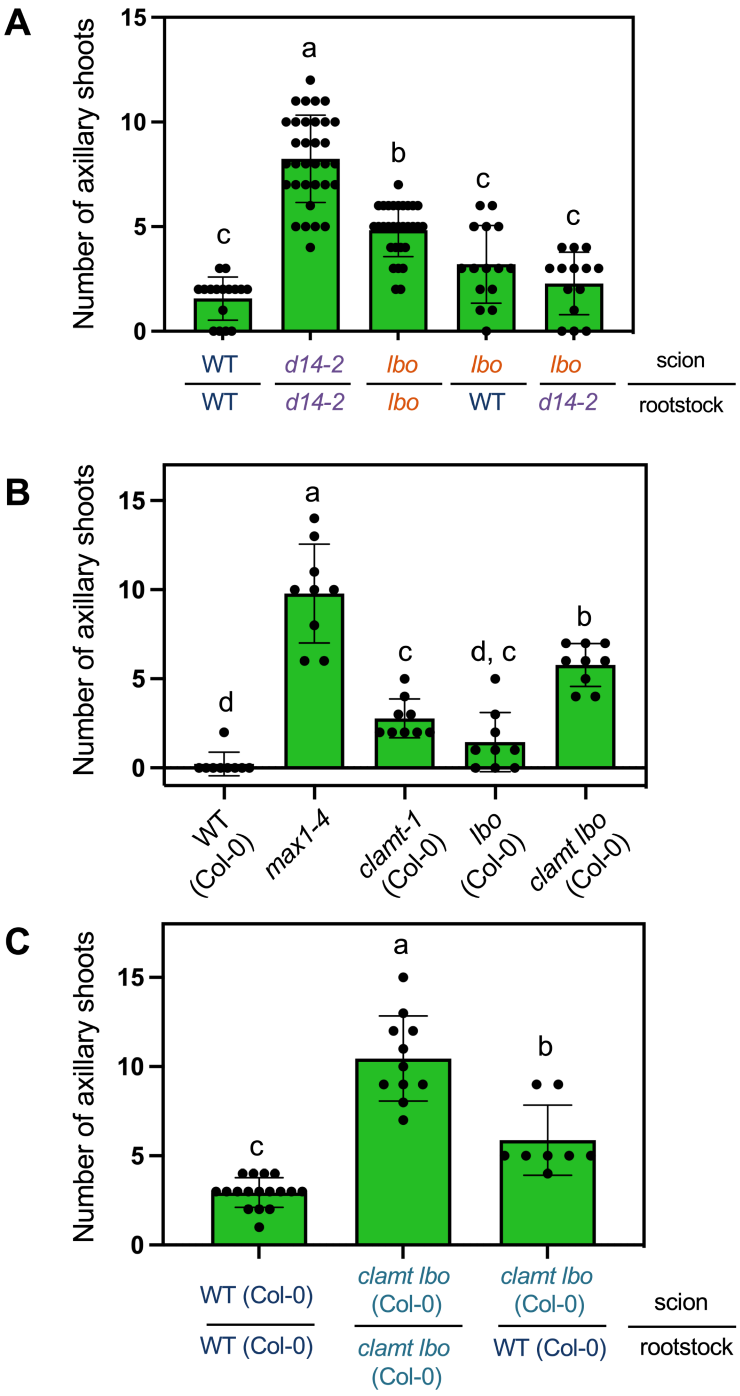


**Fig. S8**. Grafting experiments using the *lbo* and *clamt lbo* mutants. (A) The number of axillary shoots of 77-day-old grafted plants using WT, the *lbo* mutant, and the *atd14* mutant in the Col-0 background. The number of axillary shoots (> 5 mm) per plant is shown as the mean ± SD (*n* = 14-33). (B) Shoot branching phenotype of WT, *max1-4*, *clamt-1*, *lbo*, and *clamt lbo* plants in the Col-0 background. The number of axillary shoots (> 5 mm) per plant of 56-day-old plants is shown as the mean ± SD (*n* = 9). (C) The number of axillary shoots of 70-day-old grafted plants using WT (Col-0) and the *clamt lbo* mutant. The number of axillary shoots (> 5 mm) per plant is shown as the mean ± SD (WT/WT, *n* = 17; *clamt lbo*/*clamt lbo*, *n* = 11; *clamt lbo*/WT, *n* = 8). Different letters indicate significant differences at P < 0.05, Tukey's HSD.

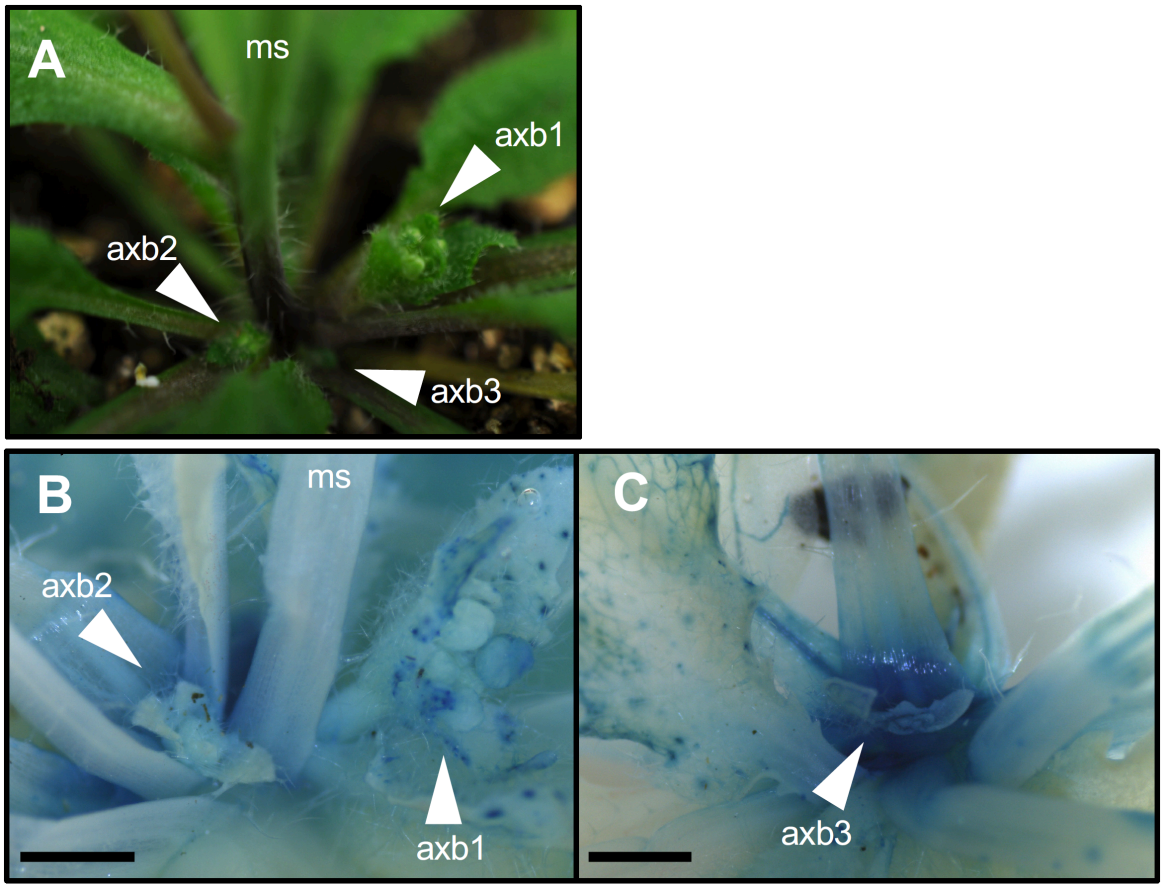


**Fig. S9.** The expression of *CLAMTpro*::*GUS* in the 40-day-old transgenic line. The GUS staining was performed for 20 h. Images from the basal part of the main stem (ms) of the same plant before staining (A) and after staining (B, C) are shown. The bar indicates 1 mm. axb; axillary bud.

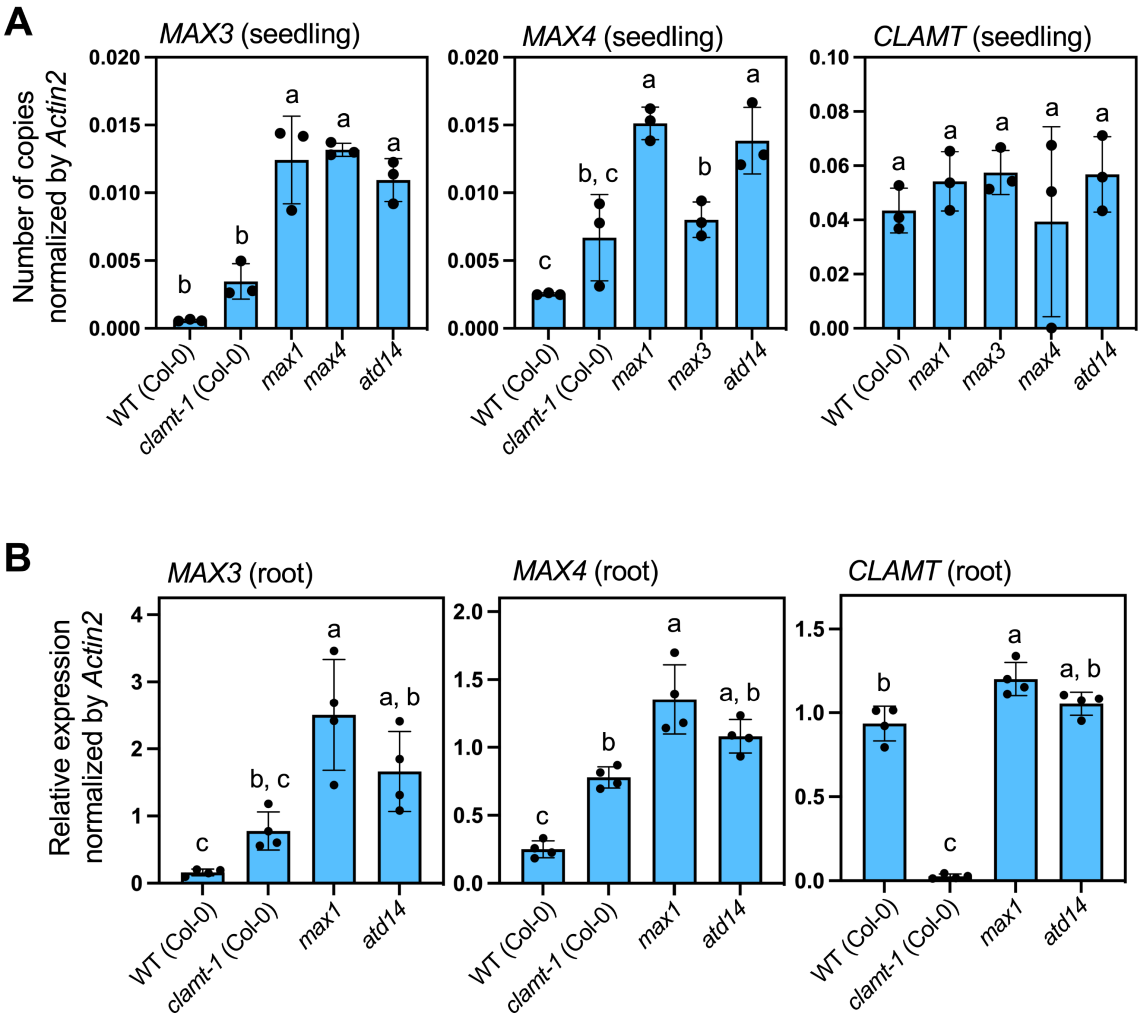


**Fig. S10**. Transcript levels of *MAX3*, *MAX4*, and *CLAMT* in the Arabidopsis SL-related mutants. Transcript levels of each gene in 14-day-old whole seedlings (A) and roots of 14-day-old seedlings (B) are shown. Transcript levels were normalized using *ACTIN2*. Data are the means ± SD [*n* = 3 (A) or *n* = 4 (B)]. Different letters indicate significant differences at P < 0.05, Tukey's HSD. In A, the *clamt-1* mutant after backcrossing with Col-0 WT four times was used.

**Table S1.**

Primers used in this study.

| Experiment | Gene ID | Primer Name | Sequence (5' to 3') |
|---|---|---|---|
| Promoter::GUS fusion | *CLAMT* | CLAMTpro-F-attB1 | ACAAGTTTGTACAAAAAAGCAGGCTTGCTAATTTGCTTTAATAAAGCTCTG |
| | | CLAMTpro-R-attB2 | ACCACTTTGTACAAGAAAGCTGGGTAGACACTCATCACAATAACAATAAAAGTGAAGAAA |
| Protein expression | *CLAMT* | CLAMT-F-BamHI | AAACCCGGGATGGATAAGAAGGATATGGAG |
| (*E.coli*) | | CLAMT-R-SalI | GGGGGATCCTCAGAGCTTCTTTCTTAGGAC |
| Protein expression | *CLAMT* | Agro-NocI_At4g36470_F | CATGCCATGGCAATGGATAAGAAGGATA |
| (*N. benthamiana*) | | Agro-NocI_At4g36470_R | ATTTGCGGCCGCTCAGAGCTTCTTTCTTA |
| qRT-PCR | *MAX3* | qRT-F | TTGCAACGCTGAAGATATGC |
| | | qRT-R | AGTATCCGTGAATGCCCAAT |
| | | TaqMan probe | [HEX]CTTCACATTTTGTGAGTATGATTCGG[BHQ1] |
| | *MAX4* | qRT-F | TATGCTCCGTCTCGATACCC |
| | | qRT-R | GATGCTGCACATATCCATCG |
| | | TaqMan probe | [HEX]CGATGCTAGGATCGGGAGATTC[BHQ1] |
| | *CLAMT* | qRT-F | CCCTGAAAACACCATCCACT |
| | | qRT-R | TATGGACTTGCCTTGCTCGT |
| | | TaqMan probe | [HEX]CTTACACTGGCTTTCCAAGGTTCCTAC[BHQ1] |
| | *ACTIN2* | qRT-F | CTTGTTCCAGCCCTCGTTTGTG |
| | | qRT-R | CCTCATCATACTCGGCCTTGG |
| | | TaqMan probe | [FAM]CTTTCAGGTGGTGCAACGACCTTAATCT[TAMRA] |
| Genotyping of *clamt* mutants | *CLAMT* | CLAMT-F-BamHI | AAACCCGGGATGGATAAGAAGGATATGGAG |
| | | CLAMT-R-SalI | GGGGGATCCTCAGAGCTTCTTTCTTAGGAC |
| | | Transposon 5-1a | ACGGGATCCCGGTGAAACGGT |
| | | pst10798-priA | ACAAGCATTCATGACCACCA |

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