# Dual biosynthetic pathways to phytosterol via cycloartenol and lanosterol in *Arabidopsis*

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**The differences between the biosynthesis of sterols in higher plants and yeast/mammals are believed to originate at the cyclization step of oxidosqualene, which is cyclized to cycloartenol in higher plants and lanosterol in yeast/mammals. Recently, lanosterol synthase genes were identified from dicotyledonous plant species including *Arabidopsis*, suggesting that higher plants possess dual biosynthetic pathways to phytosterols via lanosterol, and through cycloartenol. To identify the biosynthetic pathway to phytosterol via lanosterol, and to reveal the contributions to phytosterol biosynthesis via each cycloartenol and lanosterol, we performed feeding experiments by using [6-$^{13}C^2H_3$]mevalonate with *Arabidopsis* seedlings. Applying $^{13}C$-{$^1H$}{$^2H$} nuclear magnetic resonance (NMR) techniques, the elucidation of deuterium on C-19 behavior of phytosterol provided evidence that small amounts of phytosterol were biosynthesized via lanosterol. The levels of phytosterol increased on overexpression of *LAS1*, and phytosterols derived from lanosterol were not observed in a *LAS1*-knockout plant. This is direct evidence to indicate that the biosynthetic pathway for phytosterol via lanosterol exists in plant cells. We designate the biosynthetic pathway to phytosterols via lanosterol "the lanosterol pathway." *LAS1* expression is reported to be induced by the application of jasmonate and is thought to have evolved from an ancestral cycloartenol synthase to a triterpenoid synthase, such as $\beta$-amyrin synthase and lupeol synthase. Considering this background, the lanosterol pathway may contribute to the biosynthesis of not only phytosterols, but also steroids as secondary metabolites.**

metabolic diversity | mevalonate | sterol | tracer experiment | triterpene

Sterols are indispensable compounds in all eukaryotes, because they are structural components of plasma membranes. In mammals, insects, and higher plants, sterols are also converted to steroidal hormones. Because of their chemical significance, the main pathway of sterol biosynthesis has been studied extensively, and is believed to be fully understood (1). The differences in the biosynthesis of sterols between higher plants and yeast/mammals are generally accepted to begin at the cyclization step of 2,3-oxidosqualene, a common precursor. Ergosterol and cholesterol are biosynthesized via lanosterol, catalyzed by lanosterol synthase (LAS), in yeast and in mammals (2, 3), respectively. Phytosterols, such as campesterol and sitosterol, are biosynthesized via cycloartenol and catalyzed by cycloartenol synthase (CAS) in higher plants (Fig. 1) (4, 5). Recently, however, three different laboratories have identified LAS genes from dicotyledonous plant species including *Arabidopsis thaliana* (6, 7), *Panax ginseng* (6), and *Lotus japonica* (8) using a yeast expression system. The existence of LAS genes in the plant kingdom led us to address the question of whether the biosynthetic pathway of phytosterols via lanosterol as the first cyclic intermediate exists.

To overturn the common knowledge, one must clarify that endogenous lanosterol is metabolized to phytosterols by using upstream substrates such as acetate and mevalonate (MVL).

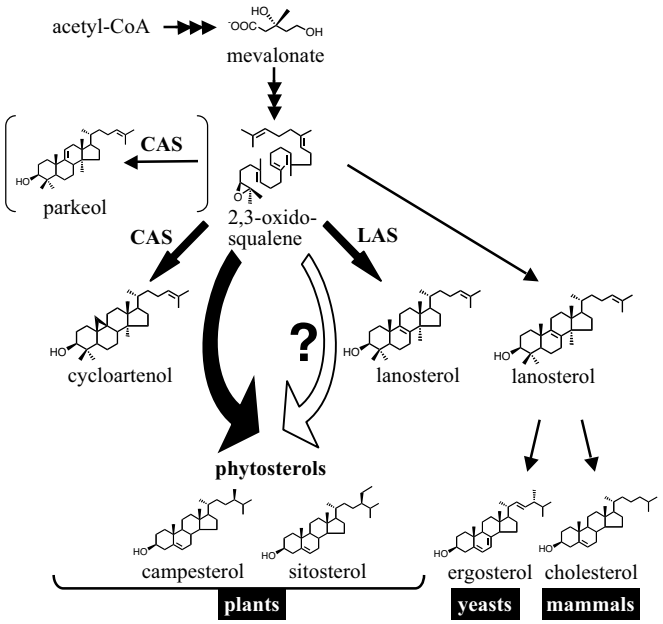


**Fig. 1.** Cyclization step of oxidosqualene in yeasts, mammals, and plants. Recently, lanosterol synthase genes were identified from several plants, *Arabidopsis thaliana*, *Panax ginseng*, and *Lotus japonica* (6–8). However, no clear data confirm the existence of that biosynthetic pathway of phytosterol via lanosterol in the plant kingdom. CAS, cycloartenol synthase; LAS, lanosterol synthase.

Both cycloartenol and lanosterol are biosynthesized from oxidosqualene after the formation of the protosteryl cation. Both H-19 and H-9 protons of the protosteryl cation are deprotonated to afford cycloartenol and lanosterol, respectively (Fig. 2) (9). We noted the difference of this cyclization mechanism, and designed a tracer experiment by using [6-$^{13}C^2H_3$]mevalonate ([6-$^{13}C^2H_3$]MVL) (Fig. 2). Each of the labeled atom positions, C-18, C-19, C-21, and C-27 (Pro-*R*), of phytosterol are biosynthetically equivalent to C-6 of MVL (10). Elucidation of the deuterium on C-19 behavior of phytosterol made it possible to clarify whether the phytosterol is biosynthesized via either

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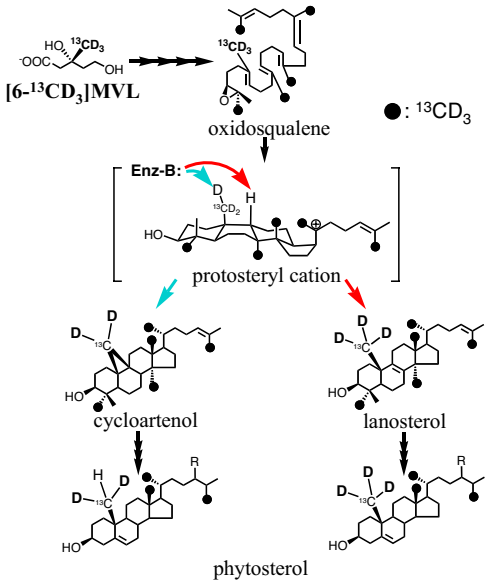


**Fig. 2.** Label pattern of phytosterol in the feeding experiment using [6-$^{13}CD_3$]MVL. C-19 of phytosterol biosynthesized via cycloartenol and lanosterol are labeled as $^{13}CD_2H$ and $^{13}CD_3$, respectively.

cycloartenol or lanosterol. If the phytosterol retained two deuterium labels at the C-19 position, then it was biosynthesized via cycloartenol. In the case when three deuteriums are detected on C-19, the results would indicate that the phytosterol was biosynthesized via lanosterol. The number of deuterium labels was analyzed by proton and deuterium double-decoupled $^{13}C$-nuclear magnetic resonance (NMR) ($^{13}C$-{$^1H$}{$^2H$}NMR). Because the signal for a carbon connected to a deuterium would be shifted theoretically by approximately 0.3 ppm upfield because of isotope effects (11), C-19 carbon peaks connected to two and three deuteriums were shifted to 0.6 ppm and 0.9 ppm upfield, respectively, compared with the unshifted $^{13}C$ spectrum. By using this strategy, one can determine whether phytosterols are biosynthesized via lanosterol. The contribution to phytosterol biosynthesis via lanosterol can also be determined by comparing the area of two $^{13}C$ peaks, namely, the signals shifted 0.6 and 0.9 ppm upfield.

In this study, [6-$^{13}C^2H_3$]MVL was synthesized and fed to the seedlings of the wild type (WT), LAS1 overexpressing plant, and a *las1* mutant of *Arabidopsis*. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of phytosterols was performed to verify the actual number of incorporated labeled carbons and deuteriums. After HPLC separation, sitosterol, the major phytosterol in *Arabidopsis*, was analyzed by $^{13}C$-{$^1H$}{$^2H$}NMR. Although the major peak indicated the presence of two retained deuterium labels, a minor peak indicative of three deuteriums was also observed from the WT seedling. This minor peak intensity was increased by 3-fold for the LAS1 overexpression seedling, and was not observed from the *las1* mutant seedling. This study provides direct evidence that the biosynthetic pathway of phytosterol via lanosterol exists in plant cells. The existential meaning of this pathway is also discussed.

## Results

**Feeding of Labeled MVL to the WT Seedling of *Arabidopsis*.** The [6-$^{13}C^2H_3$]MVL was prepared by the method of Eguchi *et al.* (12) with some modifications and is outlined in Fig. 3. The 5-day-old seedlings of the WT grown on Murashige-Skoog (MS) basal plates were cultured for 2 weeks in MS liquid media containing 1.0 mM [6-$^{13}C^2H_3$]MVL supplemented with the hydroxymethylglutaryl (HMG)-CoA reductase inhibitor, lovastatin (10 $\mu$M),

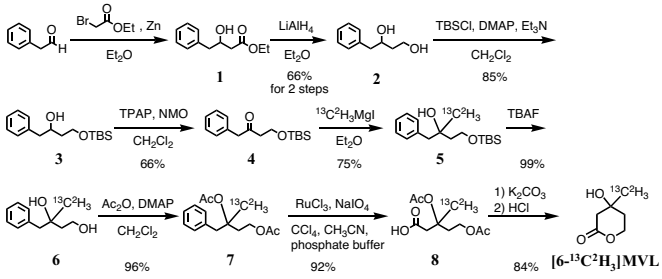


**Fig. 3.** Total synthesis of [6-$^{13}C^2H_3$]MVL.

to increase the incorporation ratio of the labeled MVL into phytosterol (13). The isolated sterol fraction was analyzed initially using gas chromatography-mass spectrometry (GC-MS). The mass spectra for major phytosterols, such as campesterol, stigmasterol, and sitosterol, are shown in Fig. 4*A*. Nonlabeled campesterol, stigmasterol, and sitosterol have a molecular ion at $m/z$ = 400, 412, and 414, respectively. The extracted phytosterols had major molecular ions at $m/z$ = 415, 427, and 429, corresponding to those molecules with four $^{13}C$ atoms and 11 $^2H$ atoms per molecule. Phytosterols were biosynthesized effectively from exogenous [6-$^{13}C^2H_3$]MVL in this experiment. GC-MS results suggested that three deuteriums at C-6 of MVL were retained in the C-18, -21, and -27 positions, whereas a deuterium at the C-19 position was substituted with a hydrogen during sterol biosynthesis. Phytosterol with three deuteriums retained at the C-19 position could not be determined by the analyses of MS data. Next, the major phytosterol, sitosterol, was analyzed by using NMR after the separation of sterol fraction by HPLC (High Performance Liquid Chromatography). The labeling pattern of the sitosterol was determined by $^{13}C$-{$^1H$}{$^2H$}NMR. The $^{13}C$-{$^1H$}{$^2H$}NMR spectrum and chemical shift values for C-18, -19, -21, and -27 are shown in Fig. 5*B* and Table 1, respectively. Resonances at 10.98 (C-18), 17.84 (C-21), 18.08 (C-27), and 18.81 (C-19) ppm were identified with 0.88, 0.94,

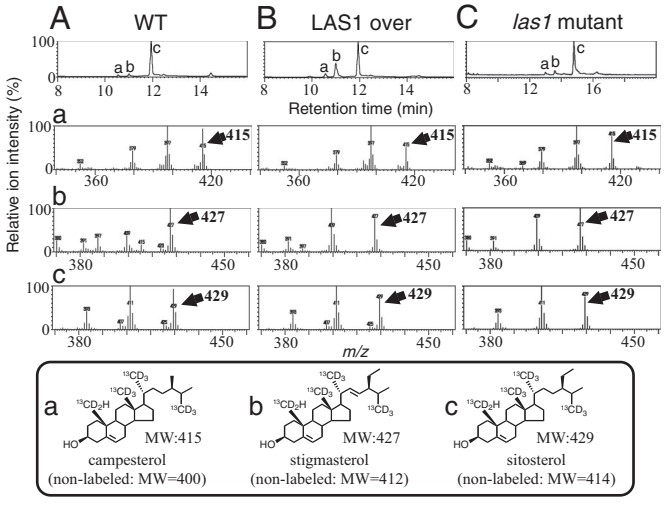


**Fig. 4.** GC chromatographs of sterol fractions and MS data for labeled phytosterols in (*A*) WT, (*B*) LAS1 overexpressing plant, and (*C*) *las1* mutant. Nonlabeled campesterol, stigmasterol, and sitosterol have molecular ions at $m/z$ = 400, 412, and 414, respectively. Extracted phytosterols (*a*) campesterol, (*b*) stigmasterol, and (*c*) sitosterol have major molecular ions at $m/z$ = 415, 427, and 429, respectively. Structures estimated by molecular ions are shown. GC analyses were performed with an HP-5 column and DB-1 column for WT and LAS1 overexpression, and *las1* mutant, respectively. MW, molecular weight. LAS1 over, LAS1 overexpressing plant.

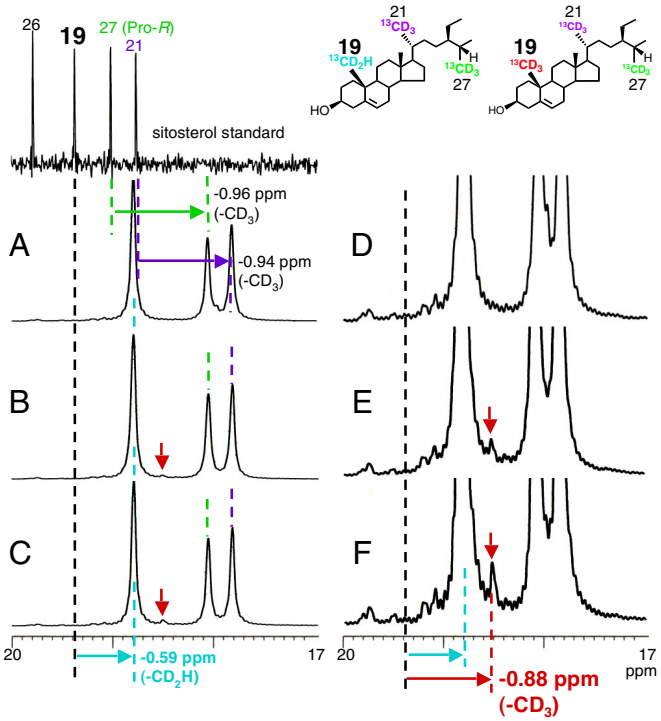


**Fig. 5.** Expanded C-19 region (17–20 ppm) of the $^{13}$C-{$^{1}$H}{$^{2}$H}NMR spectra of labeled sitosterols in (*A*) *las1* mutant, (*B*) WT, and (*C*) LAS1-overexpressing plant. Enlargements of the *y*-scale from spectra *A*–*C* are shown in *D*–*F*, respectively.

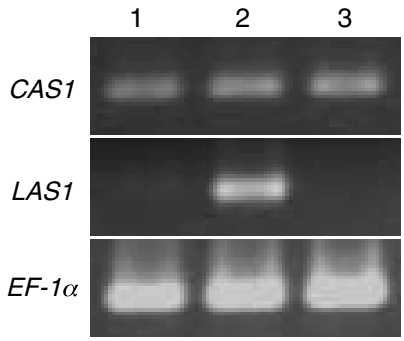


**Fig. 6.** Developmental expression profile of *CAS1* and *LAS1*. Total RNA was extracted from seedlings of (1) WT, (2) LAS1 overexpressing plant, and (3) *las1* mutant. *CAS1* and *LAS1* transcripts were amplified by 30 and 40 cycles, respectively.

0.96, and 0.59 ppm upfield shifts by comparison with resonances at 11.86, 18.78, 19.04, and 19.40 ppm of the authentic sample, respectively. These results show that C-18, -21, and -27 were labeled as $^{13}C^{2}H_3$, and C-19 was labeled as $^{13}C^{2}H_2H$. In addition, a small peak (δ 18.53) was detected shifted farther upfield by 0.28 ppm compared with the 0.59 ppm upfield shifted signal of C-19 as a minor peak (Fig. 5*E*). This peak shows that C-19 was labeled as $^{13}C^{2}H_3$. Although the C-19 atom retained two deuteriums, consistent with the intermediacy of cycloartenol, a peak indicative of three retained deuteriums was also detected, which is consistent with a lanosterol intermediate. The minor peak area corresponded to approximately 1.5% of the major peak area. To confirm the significance of this peak, labeled MVL was fed to the *LAS1* overexpressing and *las1* mutant seedlings of *Arabidopsis* and this minor peak was examined.

**Feeding Labeled MVL to the Seedlings of the LAS1 Overexpressing Plant and *las1* Mutant of *Arabidopsis*.** Transgenic *Arabidopsis* lines overexpressing *LAS1* under the control of cauliflower mosaic virus 35S promoter were generated. Segregation analysis of kanamycin resistance indicated that several independent lines contained a single T-DNA insertion and were homozygous with respect to the transgene. Expression of *LAS1* in each line was analyzed by quantitative RT-PCR. The line designated L4, which showed the highest expression of *LAS1* (data not shown), was selected for further characterization. Despite the higher expression levels of *LAS1*, no visible phenotype was observed in the L4 plant, and the sterol profile of L4 was comparable to that of the WT (data not shown). Developmental expression profiles of *CAS1* and *LAS1* in WT, LAS1 overexpressing plant, and *las1* mutant were shown in Fig. 6. Feeding experiments using L4 and the *las1* mutant (6) were also performed. Similar to the feeding experiment using the WT, GC-MS analysis of the major phytosterols indicated that labeled MVL was incorporated efficiently into major phytosterols such as campesterol, sitosterol, and stigmasterol (Fig. 4 *B* and *C*). Similar to the WT, the major peaks for L4 and *las1* showed that two deuteriums were retained at C-19. Thus, the GC-MS analyses were insufficient to conclude whether three deuteriums were retained at the C-19 position. Sitosterol was purified using HPLC and was subjected to analysis by $^{13}$C-{$^{1}$H}{$^{2}$H}NMR (see Table 1 and Fig. 5 *C* and *F*). Although the major peak for C-19 indicated two retained deuteriums, similar to results for the WT seedling, a minor peak indicative of three retained deuteriums was observed. The intensity of the focused peak (δ 18.53) in L4 was approximately three times greater than that for the WT. The minor peak area corresponded to approximately 4.5% of the major peak area. In addition, no peak at δ 18.53 was detected from feeding [6-$^{13}C^{2}H_3$]MVL to the *las1* mutant (Table 1 and Fig. 5 *A* and *D*). These data clearly show that the biosynthetic pathway of phytosterol via lanosterol exists in *Arabidopsis*. Hereafter, the bio-

**Table 1. $^{13}$C-NMR spectra of C-18, 19, 21, and 27 of sitosterol biosynthesized from [6-$^{13}CD_3$]MVL in *Arabidopsis* seedlings**

| | Sitosterol $\delta_C$ ($^{1}\Delta\delta_C$)* | | | |
|---|---|---|---|---|
| Carbon | Standard | WT | LAS1 over† | *las1* mutant |
| C-18 | 11.86 | 10.98 (−0.88) | 10.98 (−0.88) | 10.98 (−0.88) |
| C-19 | 19.40 | 18.81 (−0.59) | 18.81 (−0.59) | 18.81 (−0.59) |
| | | 18.53 (−0.87), 1.5%† | 18.52 (−0.88), 4.5% | Not detected |
| C-21 | 18.78 | 17.84 (−0.94) | 17.84 (−0.94) | 17.84 (−0.94) |
| C-27 | 19.04 | 18.08 (−0.96) | 18.08 (−0.96) | 18.08 (−0.96) |

*The magnitude of the α-deuterium isotope shift ($^{1}\Delta\delta_C$) is shown in parenthesis.
†The relative value shown as % was calculated by comparing the peak intensities for two and three retained deuteriums.
‡LAS1 overexpressing plant

synthetic pathways of phytosterol via lanosterol and cycloartenol are referred to as the lanosterol and cycloartenol pathways, respectively.

**Complementation Test of the *cas1-2* Mutant by *LAS1*.** Next, we investigated whether the lanosterol pathway contributes to plant development. Recently, *cas1*-knockout alleles, *cas1-2* and *cas1-3*, were identified to be male gametophyte-lethal (14). The *cas1-2* heterozygous plants were pollinated with pollen from the transgenic L4 plants to examine whether the overexpressed *LAS1* gene compensates for the *cas1*-knockout mutation. However, *cas1-2*-homozygous plants could not be obtained in the F2 generation. To investigate why *cas1*-homozygous plants were not obtained in the L4 background, we examined the expression levels of *CAS1* and *LAS1* by using RT-PCR analysis. The expression levels of *CAS1* were nearly equal among the WT, L4, and *las1* mutant. The weak *LAS1* expression observed in the WT was induced strongly in L4. Unexpectedly, the expression level of *LAS1* in L4 was much less than that of *CAS1* (Fig. 6). The lethal phenotype of *cas1-2*, even in the L4 background, may therefore be related to the lower expression level of *LAS1*.

## Discussion

The feeding experiments using [6-$^{13}C^2H_3$]MVL demonstrated the hydrogen behavior on the C-19 position of phytosterol over the course of its biosynthesis. This study provides direct evidence that the lanosterol pathway exists as a sterol biosynthetic pathway in *Arabidopsis*. In the 1960s and 1970s, controversy existed as to whether lanosterol, together with cycloartenol, are the first cyclic intermediates in phytosterol biosynthesis. Although [$^{14}$C]cycloartenol and [$^{14}$C]lanosterol were converted to phytosterol in many plants and callus (15, 16), [$^{14}$C]acetate and/or [$^{14}$C]mevalonate were incorporated only into cycloartenol, and lanosterol had not been identified from the plants (15, 17). The conversion of lanosterol into phytosterols may be because of a lack of specificity of the enzymes responsible for methylation at C-24 and demethylation at C-4 (15, 17). These findings support the conclusion that the biosynthetic intermediate of phytosterol was just cycloartenol, and that lanosterol was not accepted as the intermediate.

Another approach taken to identify the first cyclic compound in sterol biosynthesis was that of Seo and coworkers, who demonstrated that the C-19 atom of cholesterol in dog hepatocytes (18) and ergosterol in yeast (19) retain three deuteriums, whereas phytosterol in cultured cells of higher plants retains two deuteriums (20); this is consistent with the intermediacy of lanosterol and cycloartenol detected by the feeding experiment using [2-$^{13}C^2H_3$]acetate with $^{13}$C-{$^1$H}{$^2$H}NMR analysis. However, the signals for the C-19 atom of the cholesterol, ergosterol, and phytosterol with one and two retained deuterium labels were also detected. Because the deuteriums at the C-2 position of the acetate are diluted with protons during the biosynthesis from acetate to oxidosqualene, likely by keto-enol tautomerization of acetoacethyl-CoA, the feeding experiments using [2-$^{13}C^2H_3$]acetate are insufficient to compare the intensities of the peaks for two and three retained deuteriums. The H-6 of mevalonic acid is not diluted with other protons during the biosynthesis of oxidosqualene from mevalonic acid (21, 22). In the feeding experiments using [6-$^{13}C^2H_3$]MVL, three deuteriums were retained on C-18, -21, and -27 of sitosterol. Deuterium substitution on C-19 occurred only in the cyclization step of oxidosqualene. In this study, the peak corresponding to three retained deuteriums was observed using $^{13}$C-{$^1$H}{$^2$H}NMR, despite weak intensity of the peak. These results counter beliefs held for approximately 40 years.

Lanosterol has been identified from several plant extracts including the latex of many *Euphorbia* plants (23–26), but whether lanosterol is the first cyclic intermediate in phytosterol biosynthesis in these plants is controversial. Because labeled cycloartenol was converted to lanosterol in the latex, but not vice versa (27), lanosterol was not believed to be the first cyclic intermediate. However, it has been also reported that no conversion of cycloartenol to lanosterol has been demonstrated using the latex of *Euphorbia lathyris* (25). Thus, whether *Euphorbia* plants with lanosterol have the lanosterol pathway has not been established. This argument may be resolved by means of a tracer experiment using the [6-$^{13}C^2H_3$]MVL. Although this study found the lanosterol pathway only in *Arabidopsis*, one may clarify whether the lanosterol pathway exists generally in the plant kingdom by performing the tracer experiment using identified *LAS* genera, *Panax* and *Lotus*. Additionally, other plant species, for example, those of *Euphorbia*, *Ochromonas*, *Nicotina*, *Pinus*, and *Pea*, in which the lanosterol pathway has not been identified (15) can also be examined.

When *Arabidopsis CAS1* is expressed in yeast, a small amount of 9,24-lanostadien-3$\beta$-ol (parkeol), was detected (1%) in addition to cycloartenol (99%) (28). Parkeol is also biosynthesized from oxidosqualene after formation of a protosteryl cation. The H-11 proton of the protosteryl cation is deprotonated to afford parkeol. Therefore, if phytosterol is biosynthesized from parkeol, three deuteriums should be retained at the C-19 position of phytosterol. Because the peak for three retained deuteriums was not detected in the feeding experiment by using the *las1* mutant, and yet the sitosterol retained three deuteriums at C-19, we conclude that the phytosterol was not biosynthesized via parkeol but rather via lanosterol in the WT plant (Fig. 1). These results are consistent with a previous report indicating that parkeol is not incorporated into phytosterol (29).

Cycloartenol is metabolized into sitosterol via obusifoliol (4$\alpha$,14$\alpha$-dimethyl- ergosta-8,24(28)-dien-3$\beta$-ol), which is biosynthesized from cycloartenol after C-24 methylation, C-4 demethylation, and cyclopropyl isomerization. In the biosynthesis of phytosterol via lanosterol, one may reasonably surmise that lanosterol is metabolized into obusifoliol after C-24 methylation and C-4 demethylation. However, Pascal and coworkers reported that a microsome preparation isolated from maize contains the enzymatic system that functions in the oxidative C-4-mono-demethylation of 4,4-dimethylsterols (30). Substrate specificity of the system was identified by incubations of the microsome by using various 4,4-dimethylsterols. In these assays, the presence of a 9$\beta$,19-cyclopropane ring is compulsory for the demethylation. In *Arabidopsis*, substrate specificity of the enzymes for C-4 demethylation may be different from those in maize. Alternatively, a specific biosynthetic pathway for lanosterol metabolism may exist in *Arabidopsis*.

In higher plants, isoprenoids are produced not only via the cytosolic MVA pathway but also via the plastidic methylerythritol phosphate (MEP) pathway (31). Labeling experiments have shown that sesquiterpenoids, triterpenoids, and sterols mainly comprise isoprene units, namely isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), via the classical MVA pathway (31). Despite the compartmentation of these two pathways, metabolic flow between them has been reported (32, 33). Kasahara *et al.* reported that when *Arabidopsis* were fed labled precursors, the contribution of the MEP pathway to phytosterol biosynthesis was several percentages (13). In our tracer experiments of [6-$^{13}C^2H_3$]MVL, the contribution of the isoprene units via the MEP pathway could not be determined. By mass spectrum analyses of phytosterols (Fig. 4), the incorporation rate of the labeled MVL to phytosterols was approximately estimated to be 95%, which means almost all phytosterols are biosynthesized from the labeled MVL. From these results, we speculate that under our experimental conditions the contribution of the MEP pathway to phytosterol biosynthesis is minimal. However, Dudareva and coworkers reported that the MEP pathway supports sesquiterpene formation in snapdragon flowers, suggesting that the trafficking of IPP occurs unidirectionally

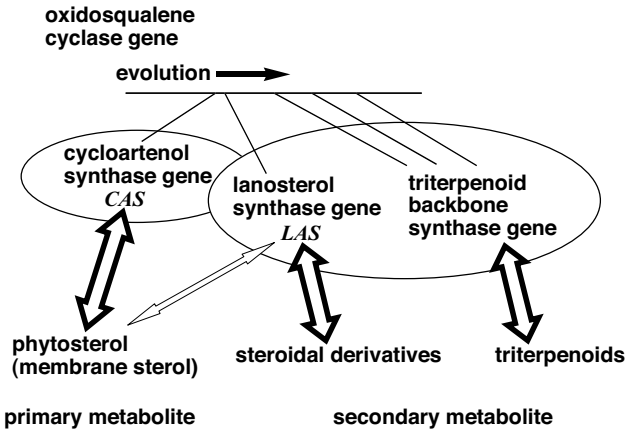


**Fig. 7.** A hypothesis concerning the function of LAS.

from plastids to cytosol (34). Moreover, when the MVA pathway was shut down by HMG-CoA reductase (HMGR) inhibitor in tobacco bright yellow-2 cells, the MEP pathway became the only IPP and DMAPP source of phytosterol biosynthesis (32). We think that the contribution of isoprene units from the MEP pathway to phytosterol biosynthesis either via cycloartenol or via lanosterol can be elucidated through the tracer experiment by using labeled precursors of the MEP pathway such as 1-[$^{13}C^{2}H_3$]-1-deoxy-D-xylulose or 2-*C*-[$^{13}C^{2}H_3$]methyl-D-erythritol-4-phosphate. It will be interesting to find out whether the contribution of the MEP pathway to dual sterol backbone synthesis will be the same as that of the MVA pathway.

The pathway to sitosterol via lanosterol was revealed, and its contribution to sitosterol biosynthesis was 1.5% relative to the cycloartenol pathway in *Arabidopsis* seedlings. The contribution of the lanosterol pathway to sitosterol was increased from 1.5% to 4.5% by *LAS1* overexpression with the 35S promotor. Unlike the ubiquitous expression of *CAS1* throughout plant development, the expression of *LAS1* is different in each plant tissue (6). *LAS1* is expressed at high levels in the siliques and stems, and at low levels in seedlings and leaves (6) (Fig. 6). From these results, it is conceivable that the contribution of the lanosterol pathway to phytosterol is different in each plant tissue. However, homozygote plants of *cas1-2* knock out mutant alleles were not isolated from the hetrozygote parents. Overexpression of *LAS1* did not complement the male gametophyte lethality of *cas1*. Moreover, no visible phenotypes were observed in the mature plant of LAS1 overexpression and *las1* mutant, and sterol profiles of these plants were comparable to that of the WT under normal growth conditions. These results indicate that the lanosterol pathway is thought to be unnecessary to the biosynthesis of membrane sterols for cell maintenance under normal conditions. The expression of *LAS1* is induced by treatment with methyl jasmonate and *Pseudomonas* infection (35). These data suggest that the products of the lanosterol pathway may be secondary metabolites and related to plant defense compounds. Some steroids and triterpenoids are known to be involved in plant defense mechanisms. Oxidosqualene cyclases for triterpenoids such as $\beta$-amyrin and lupeol may have evolved from an ancestral CAS (36). In the course of molecular evolution, plant LAS has been considered to be located between CAS and triterpenoid synthase (8) (Fig. 7). There were qualitative and quantitative differences in the triterpenoid concentrations among the tissues; in particular, approximately 200 times as much total triterpenoids were found in the siliques as in the seedling. Conversely, no appreciable differences in sterol content were found (K.O., M.S., T.M., unpublished results). These data suggest that, unlike sterols, triterpenoids are not indispensable metabolites to cell function, but have characteristic functions in each tissue. Lanosterol metabolites may also be dispensable in relation to cell function under normal conditions, but have characteristic functions in each tissue for stress conditions. We might identify a 1.5% contribution of the lanosterol pathway to sitosterol as a biosynthetic intermediate for a lanosterol metabolite, but not membrane sitosterol, in the seedling at basal conditions. Analyses using plant species containing various kinds of steroids will provide clues as to the contribution of the lanosterol pathway to steroid biosynthesis. Understanding the relevance of the lanosterol pathway in plant cells will lead to unraveling the complicated biosynthetic mechanisms of sterols and steroids.

## Materials and Methods

**Synthesis of [6-$^{13}C^{2}H_3$]MVL.** See Fig. 3 and the supporting information (SI) *Text*.

**Construction of the LAS1 Overexpression Gene for *Arabidopsis*.** The LAS1 containing fragment from digestion of pLAS1 (6) with *Bam*HI and *Xho*I was ligated into similarly digested pENTR1A vector (Invitrogen). The resultant entry clone was used for the *att*L × *att*R (LR) recombination reaction between GATEWAY-converted binary vector pBCR82 (H. Seki *et al.* unpublished data).

**Plant Material.** *Arabidopsis thaliana* (L.) Heynh. ecotype Col, LAS1 overexpressing plant (Col background) and *las1* (Col background) were germinated on agar plates containing MS medium (Gibco) and 1% sucrose for analyzing the expression profiles of *CAS1* and *LAS1*.

**Feeding of [6-$^{13}C^{2}H_3$]MVL to Lovastatin-treated Seedlings.** Seedlings were grown for 5 days on MS agar media before transfer to MS liquid media (1% sucrose). Immediately after the transfer, lovastatin (MeOH solution, final concentration 10 $\mu$M) and [6-$^{13}C^{2}H_3$]MVL (filter-sterilized $H_2O$ solution, final concentration 1 mM) were added aseptically to the liquid media, and the plants were grown for 15, 17, and 15 days for the WT, LAS1 overexpressing plant, and *las1* mutant, respectively.

**Tissue Extractions and Sitosterol Isolations.** Freeze-dried plant tissues (WT 5.5 g, LAS1 overexpressing plant 6.0 g, *las1* mutant 5.8 g) were extracted three times with $CHCl_3$-MeOH (1:1). After addition of Celite to the extract, the solvents were removed under reduced pressure and the extracts were adsorbed onto Celite. The adsorbed samples were placed in silica gel chromatography, and eluted with hexane-EtOAc (2:1) and $CHCl_3$-MeOH (1:1). The hexane-EtOAc eluent was dried and saponified with 10 ml of each of MeOH and 20% KOH aq. for 1 h at 80°C. The $CHCl_3$-MeOH eluent was dried in a rotary evaporator. The residue and extraction debris were combined and hydrolyzed with 10 ml of each of MeOH and 4 N HCl for 1 h at 80°C. These reaction mixtures were then extracted three times with 20 ml of hexane, and the combined hexane layer was evaporated to dryness. A small portion of the residue was dissolved in acetone and analyzed by following GC-MS. The residue was separated using silica gel chromatography with hexane-EtOAc (5:1). The fractions containing sterols were combined, further separated on silica gel preparative TLC plates, and developed twice with hexane-EtOAc (5:1) to give a purified phytosterol fraction. Phytosterol fraction was separated by reverse phase HPLC (column, Nakarai, 25 cm × 2 mm i.d.; solvent, $CH_3CN/H_2O$ (95:5); flow rate, 1.0 ml $min^{-1}$; UV detector at 210 nm). In HPLC separation, fractions were collected automatically (retention time: 18–25 min, 40 sec/fraction). An aliquot of all fractions were evaluated for purity using GC-MS. We corrected the fractions of single peak of sitosterol, and the solvent was removed under reduced pressure, followed by $^{13}C$-{$^{1}H$}{$^{2}H$}NMR analysis.

**Analytical Methods.** The $^{1}H$ and $^{13}C$-{$^{1}H$}NMR, and $^{13}C$-{$^{1}H$}{$^{2}H$}NMR spectra were recorded on Bruker DSX-300 and DRX-700 spectrometers operated at 298 K, respectively. All NMR samples were dissolved in $CDCl_3$. $^{1}H$ and $^{13}C$ NMR chemical shifts were reported in $\delta$ values based on internal TMS ($\delta_H = 0$) and a solvent signal ($CDCl_3$ $\delta_C = 77.0$) as the reference. GC-MS analysis was carried out on a mass spectrometer (JMS-AM SUN200, JEOL) connected to a gas chromatograph (6890A, Agilent Technologies) with a capillary column HP-5 (30 m × 0.32 mm, 0.25 $\mu$M film thickness, J&W), DB-1 (30 m × 0.25 mm, 0.25 $\mu$M film thickness, J&W). The analytical conditions of GC-MS were the same as described in ref. 37.

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