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# Sterol composition and growth of transgenic tobacco plants expressing type-1 and type-2 sterol methyltransferases

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**Abstract.** Transgenic tobacco (*Nicotiana tabacum* L.) plants with altered sterol composition were generated by transformation with plant cDNAs encoding type-1 and type-2 sterol methyltransferases (SMTs; EC 2.1.1.41). For both SMT1 and SMT2 transformants, the transformation was associated with a reduction in the level of cholesterol, a non-alkylated sterol. In SMT1 transformants a corresponding increase of alkylated sterols, mainly 24-methyl cholesterol, was observed. On the other hand, in SMT2 transformants the level of 24-methyl cholesterol was reduced, whereas the level of sitosterol was raised. No appreciable alteration of total sterol content was observed for either genotype. The general phenotype of transformants was similar to that of controls, although SMT2 transformants displayed a reduced height at anthesis. The results show that plant sterol composition can be altered by transformation with an SMT1 cDNA without adverse effects on growth and development, and provide evidence, in planta, that SMT1 acts at the initial step in sterol alkylation.

**Key words:** Cholesterol – *Nicotiana* (sterol metabolism) Sterol metabolism – Sterol methyltransferase – Transgenic tobacco

## Introduction

Sterols are components of membranes and have a function in regulating membrane fluidity and permeability. Plants always contain a mixture of sterols, commonly with 24-ethyl sterols such as stigmasterol and sitosterol as the dominating species. Additional plant sterols are 24-methyl cholesterol (a mixture of the 24$\alpha$ and 24$\beta$ epimers campesterol and 22-dihydrobrassicasterol, respectively), and in some families, e.g. the Solanaceae, cholesterol, a sterol without alkylation at C24 (Hartmann 1998). The 24-alkylations, which are typical of sterols from fungi and higher plants, are performed by two *S*-adenosyl-L-methionine:sterol C24-methyltransferases (SMTs). In the first step, cycloartenol is methylated to 24-methylene cycloartanol (Russel et al. 1967; Wojciechowski et al. 1973; Rahier et al. 1984; Nes et al. 1991). In the second step, 24-methylene lophenol is methylated to 24-ethylidene lophenol (Fontenau et al. 1977). Recently, sequence analysis of SMT cDNAs from a variety of plant species has shown the existence of two distinct classes of SMTs, denoted type 1 and type 2 (Grebenok et al. 1997; Bouvier-Navé et al. 1998). The enzymatic identity of the two classes was shown by the cloning of SMT type 1 (SMT1) and type 2 (SMT2) from tobacco and their expression in a yeast mutant lacking SMT activity (Bouvier-Navé et al. 1998). Analysis of sterol composition and substrate specificity of SMT in the transformed yeast cells showed that SMT1 methylates cycloartenol, whereas SMT2 methylates 24-methylene lophenol (see Fig. 1). Moreover, the function in vivo of SMT2 has been studied by transformation of tobacco plants with an arabidopsis SMT2 cDNA (AtSMT2) expressed from the 35S promoter (Schaller et al. 1998). In transformed plants overexpressing SMT2 the ratio of sitosterol to 24-methyl cholesterol increased drastically, in some transformants more than 40-fold. In progenies from these transformants a reduced growth rate was observed, but their general phenotype was otherwise normal.

We have been studying the in-vivo function of SMT1 by a similar approach. The SMT1 acts at the branching point between cholesterol and the 24-alkylated sterols, and is of special interest in view of the proposed role of cholesterol as the precursor for various secondary compounds with a steroid skeleton, such as the poisonous glycoalkaloids in potato (Bergenstråhle et al. 1996). Furthermore, reports from studies of sterol composition and SMT activity in potato slices (Hartmann and Benveniste 1974; Bergenstråhle et al. 1993) and of

Abbreviation: SMT = *S*-adenosyl-L-methionine:sterol C24-methyl transferase

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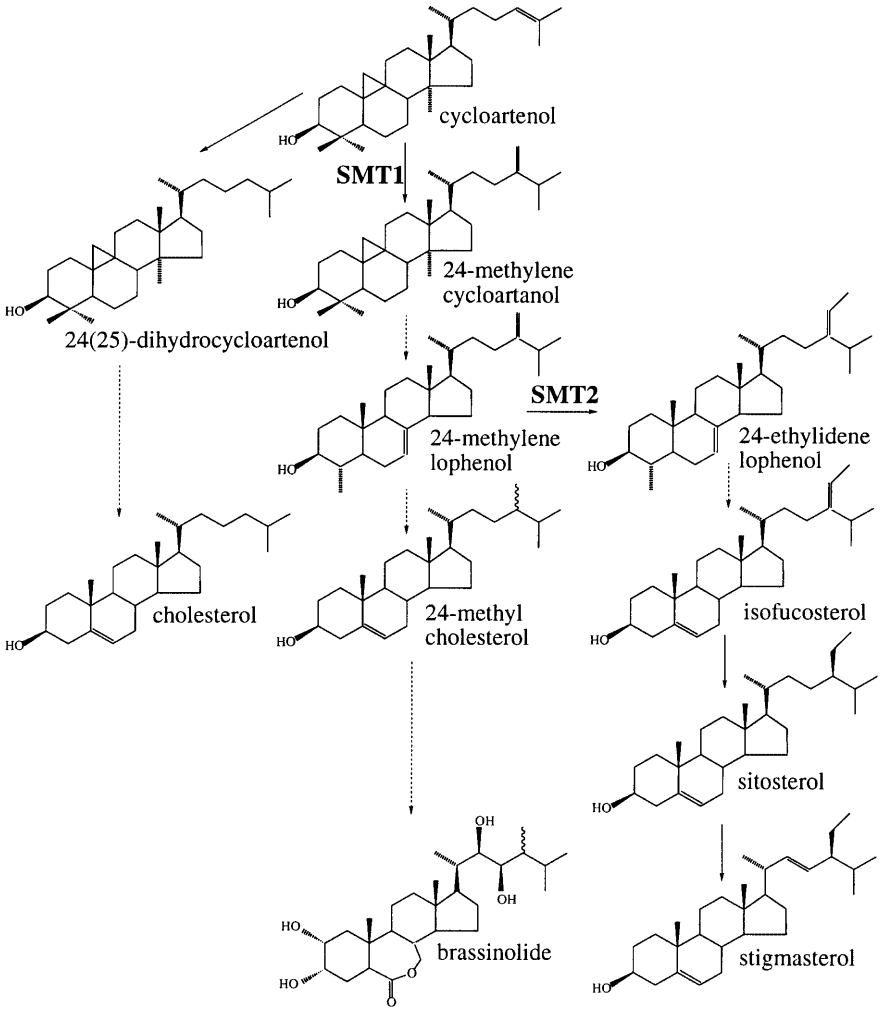


**Fig. 1.** Schematic presentation of proposed sterol biosynthesis pathways in solanaceous plants. *Dashed arrows* indicate more than one enzymatic step. The steps catalysed by sterol methyltransferases of type 1 (SMT1) and type 2 (SMT2) are indicated

transgenic tobacco plants overproducing sterols as a consequence of increased expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Schaller et al. 1995) indicate that SMT under the experimental conditions has a rate-regulatory role in the overall biosynthesis of sterols.

Here, we report on the transformation of tobacco plants with SMT1 cDNA from soybean (GmSMT1), and the effects of SMT1 overexpression on the total sterol content and sterol composition in leaves, and on growth rate of transformed plants. The work was carried out with parallel analyses of plants transformed with AtSMT2.

## Material and methods

*Vector constructions and generation of transgenic plants*

A cDNA fragment spanning the GmSMT1 coding sequence was obtained by polymerase chain reaction (PCR) on plasmid pSpm482 (Shi et al. 1996), with primers (5′) GTTGGTACCGGGTAA-GTTTTGTGGATG and (5′) GTTTCTAGATAATATGGAG-GAGCACTG. A cDNA fragment encoding AtSMT2 was generated by reverse transcription (RT)-PCR on mRNA isolated from seedlings of *Arabidopsis thaliana* (L.) Heynh., using the primers (5′) TTTGGTACCCTCACTCTTAACGAAAATGGA and (5′) GCTCTAGACGGAGAAATCAAGAGTAAGAT. The resulting fragments were cut at the thus-introduced *Kpn*I and *Xba*I restriction sites, and ligated into the *Kpn*I and *Xba*I sites of the Ti-plasmid Bin-Hyg-TX in sense orientation behind the TET1 promoter, a tetracycline-inducible derivative of the CaMV 35S promoter (Gatz et al. 1992). While this promoter enables tetracycline-inducible gene expression in lines co-transformed with the transcriptional repressor TetR, promoter activity in the absence of TetR is comparable to that of the 35S promoter. The plasmids AtSMT2.hyg and GmSMT1.hyg were electroporated into the *Agrobacterium tumefaciens* strain LBA4404. Tobacco (*Nicotiana tabacum* L.) cv. Wisconsin 38 was leaf-disc-transformed with the *Agrobacterium* strains, yielding a total number of 24 and 65 transgenic AtSMT2.hyg and GmSMT.hyg T1 lines, respectively, which were self-fertilised. As a control, hygromycin-resistant plants transformed with the empty vector were generated in parallel. Expression of the introduced genes was confirmed with RT-PCR and RNA gel blot analysis. Transgenic T2 lines were screened for an altered sterol composition, and two representative lines with similar cholesterol levels were used for growth measurements. The sterol composition of control plants was not different from that of the wild type.

*Plant growth conditions*

Hygromycin-resistant T2 seedlings were transferred to small pots with fertilised peat. After three weeks, plants were transferred to one-liter pots, randomised, and further grown during May to July

(Uppsala, Sweden) in a greenhouse at 20–25 °C with natural daylight supplemented with halogen lamps giving a quantum flux density of 70 μmol $m^{-2}$ $s^{-1}$. Plants were fertilised weekly with a 1/100 dilution of a liquid fertiliser (SuperbaS, Landskrona, Sweden).

*Sterol measurements*

For all plants analysed, the leaf blade was at the same occasion collected from the youngest leaf 15–20 cm in length when plants had reached a height of 35–40 cm. Samples (200 mg) were extracted, purified and sterol composition was analysed in duplicate on a gas chromatograph according to Bergenstråhle et al. (1996). To allow a quantification of the total sterol content, 100 μg of ergosterol was added as an internal standard to the samples before extraction.

*Preparation of microsomes and enzyme assay*

Microsomal fractions were isolated at 4 °C from young expanding leaves by homogenisation on ice using mortar and pestle in a buffer consisting of 100 mM potassium phosphate (pH 7.50, 0.3 M mannitol, 1 mM EDTA, 4 mM $MgCl_2$, 0.1% bovine serum albumin, and 10 mM $\beta$-mercaptoethanol. The homogenate was filtered and centrifuged at 10,000 *g* for 15 min, after which a microsomal pellet was obtained by centrifugation at 100,000 *g* for 90 min. The pellet was homogenised in 100 mM potassium phosphate buffer (pH 7.5), 20% glycerol, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM reduced glutathione, and stored at −70 °C before analysis of enzymatic activity. Protein content was measured in 2-ml assays with the bicinchoninic acid (BCA) protein reagent (Pierce Chemical Co., Rockford, Ill., USA), using bovine serum albumin as standard. Sodium dodecyl sulfate was added to 0.3% to the samples, which were diluted with water to reduce interference of lipids, and to obtain assay-compatible concentrations of buffer ingredients.

The SMT assays were carried out as described in Bergenstråhle et al. (1993) with the following changes: the assay volume of 0.6 ml contained 0.5 ml microsomal suspension (3–10 mg protein $ml^{-1}$) in sample buffer (pH 7.5), 100 μM [$^3$H]AdoMet (74 kBq $ml^{-1}$; Amersham International Inc., Little Chalfont, Buckinghamshire, UK), 100 μM lanosterol (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and 1 mg $ml^{-1}$ Tween 80 (Sigma-Aldrich Sweden AB). The incubations were carried out for 60–90 min. Sterols were localized on TLC plates by spraying with 0.1% (w/vol.) berberine in ethanol. The desmethylsterols were scraped off the plates and the radioactivity was determined using toluene:ethanol (2:1, v/v) and 0.4% 2-(4-t-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (w/v) as scintillant.

*Analysis of RNA gel blots*

Expanding leaves, ca. 10 cm in length, were collected from pools of three greenhouse-grown plants per genotype. Total RNA was extracted according to Logemann et al. (1987). Thirty micrograms was separated on formaldehyde agarose gels and transferred onto HybondN membranes (Amersham Pharmacia Biotech., Uppsala, Sweden). The full-length cDNAs of GmSMT1 and AtSMT2 were labelled using a random-priming kit (Amersham Pharmacia Biotech.) and used to probe the filters. After subsequent washes under stringent conditions (0.1 × SSC at 66 °C for 30 min; 1× SSC is 150 mM NaCl, 15 mM Na-citrate, pH 7.0), the filters were exposed to autoradiographic films (Kodak). For both probes, no cross-hybridisation to the endogenous tobacco SMT mRNAs or to each other was detected under these conditions. As a control of equal loading, the filters were re-hybridised with a 28S ribosomal RNA probe.

## Results

To investigate the role in planta of SMTs (Fig. 1) belonging to the type-1 and type-2 classes, transgenic plants were generated expressing the GmSMT1 and AtSMT2 cDNAs from the TET1 promoter, a derivative of the 35S promoter. For both types of transformant, the total sterol content in leaf tissue was similar to that of control plants transformed with the empty vector (Table 1). However, as compared with control plants, significant effects on sterol composition were observed (Table 1, Fig. 2). GmSMT1 lines displayed a reduction of cholesterol levels with a concomitant increase in the level of 24-methyl cholesterol, and to a lesser extent also the 24-ethyl sterol sitosterol, but with no major alterations of the 24-ethyl sterols stigmasterol or isofucosterol. In the AtSMT2 lines, there was a clear reduction in the level of both cholesterol and 24-methyl cholesterol, whereas the level of sitosterol was raised, and that of stigmasterol and isofucosterol was unaltered. The effect on sterol composition in the transgenic lines can be illustrated by the cholesterol:24-methyl cholesterol ratio which was 0.8, 0.2, and 0.8 for control, GmSMT1 and AtSMT2 plants respectively; or with the 24-methyl cholesterol:sitosterol ratio which was 1.1, 1.2, and 0.4. This demonstrates a different function in planta for SMTs of type 1 and type 2.

RNA gel blot analysis of the SMT mRNA level in transgenic plants revealed a single 1.5-kb GmSMT1 and AtSMT2 transcript in leaf RNA (Fig. 3). The GmSMT1 mRNA level, which varied among the different lines, was associated with an effect on sterol composition. For instance, the GmSMT1 lines 6 and 54, which had the lowest cholesterol levels among the lines analysed, 7.3 ± 3.3% and 7.5 ± 1.3%, respectively, also had the strongest GmSMT1 expression (Fig. 3). Conversely, the

**Table 1.** Total sterol content in transgenic T2 tobacco plants in relation to the cholesterol level. Leaf tissue from plants transformed with the sterol methyltransferase (SMT) type-1 and type-2 cDNAs indicated, or the empty vector (control), were analysed by gas chromatography for their relative sterol levels and total sterol content. Only cholesterol levels are shown for clarity. The number of independent lines investigated was 2, 19, and 2 for control, GmSMT1, and AtSMT2 transformants, respectively. A lower cholesterol level in the AtSMT2 lines analysed had been established in preceding experiments. Numbers in parentheses indicate the number of plants found for each cholesterol level. Mean ± SD; nd, not detected

| Cholesterol level (% of total) | Total sterol content [μg (g FW)$^{-1}$] | | |
|---|---|---|---|
| | Control | GmSMT1 | AtSMT2 |
| 4–6 | nd | 381 (1) | 329 (1) |
| 6–8 | nd | 435 ± 63 (14) | 444 (1) |
| 8–10 | nd | 408 ± 46 (26) | 384 ± 24 (3) |
| 10–12 | nd | 436 ± 64 (12) | 388 ± 60 (3) |
| 12–14 | nd | 419 ± 63 (12) | 449 ± 89 (4) |
| 14–16 | 386 ± 27 (7) | 406 ± 52 (7) | nd |
| 16–18 | 395 ± 38 (5) | 425 ± 66 (4) | nd |
| 18–20 | 381 (1) | nd | nd |
| All plants | 390 ± 30 (13) | 421 ± 57 (76) | 407 ± 67 (12) |

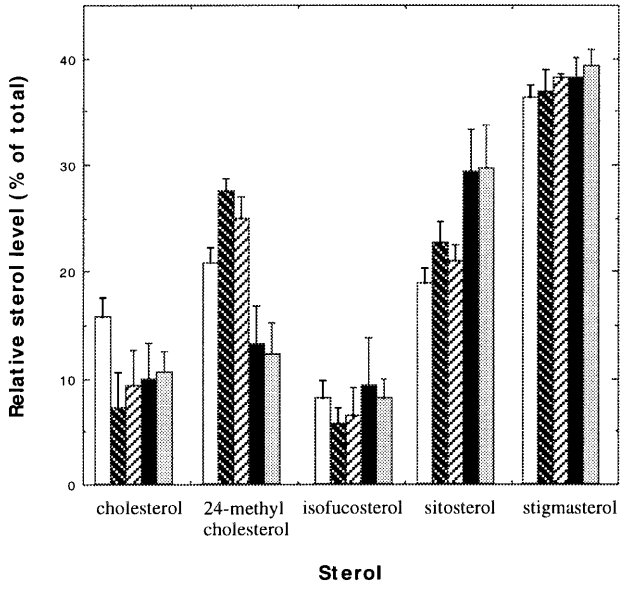


**Fig. 2.** Relative levels of the main sterols in tobacco leaf tissue. Tobacco cv. W38 was transformed with an empty Ti-plasmid (control, *white columns*), a GmSMT1 (*dark hatched columns*, line 6; *grey hatched columns*, line 51), and an AtSMT2 construct (*black columns*, line 6; *grey columns*, line 8). Sterols were extracted from leaf tissue of plants grown in parallel under greenhouse conditions and analysed by gas chromatography. $n = 13$, 3, and 6 individual plants for control, GmSMT1 and AtSMT2 lines, respectively. Mean ± SD

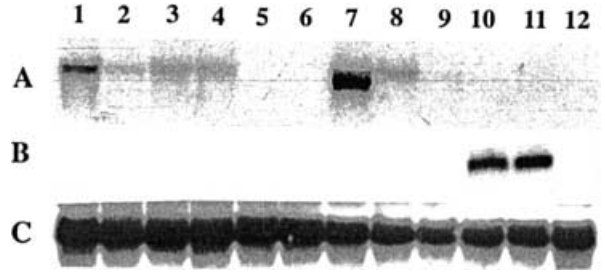


**Fig. 3.** Expression of SMT genes in transgenic tobacco plants. Total RNA in expanding leaves was extracted from pools of three plants. Thirty micrograms of RNA was separated on formaldehyde gels and transferred onto nylon filters. Filters were hybridised with probes for GmSMT1 (*A*), AtSMT2 (*B*) and 28S ribosomal RNA (*C*). Loadings from left to right: GmSMT lines 6, 8, 9, 11, 48, 52, 54, 58, 61, AtSMT lines 6, 8, and empty vector control plants

lines 52 and 61, which had cholesterol levels similar to control plants, 16.0 ± 2.3% and 15.1 ± 3.9%, respectively, had GmSMT1 mRNA levels below the detection limit (Fig. 3). Measurements of SMT activity showed that the GmSMT1 lines with a strong GmSMT1 expression (lines 6 and 54) exhibited an increase in enzyme activity, as did the AtSMT2 line 6 (Table 2). These results demonstrate that the altered sterol composition in the transgenic lines is due to expression of the transferred genes.

The general phenotype of both GmSMT1 and AtSMT2 transformants was similar to that of control plants. In AtSMT2 lines, however, growth was reduced by 17–18% (Table 3), in agreement with the study of Schaller et al. (1998), whereas no growth reduction was observed in GmSMT1 lines (Table 3).

**Table 2.** SMT activity in microsomal preparations. Microsomes were prepared from young leaves of tobacco plants at two stages of development, and SMT activity was monitored using lanosterol as the substrate. Mean value of two replicates from each sample. n.a., not analysed

| Genotype | SMT activity [fkat (mg protein)$^{-1}$] | |
|---|---|---|
| | Expt. I Seedling stage | Expt. II 15-leaf stage |
| GmSMT1 | | |
| Line 6 | 6.4 | 23.2 |
| Line 8 | n.a. | 7.8 |
| Line 48 | n.a. | 4.0 |
| Line 54 | 8.3 | n.a. |
| AtSMT2 | | |
| Line 6 | n.a. | 19.6 |
| Control | 2.9 | 15.0 |

**Table 3.** Height of transgenic tobacco plants at anthesis. Height of T2 plants transformed with the sterol methyltransferase (SMT) type-1 and type-2 cDNAs indicated, or the empty vector (control), was measured at anthesis. Mean ± SD. Numbers in parentheses indicate the number of plants measured for each line. An asterisk indicates a difference from control significant at $P < 0.001$, Students *t*-test

| Genotype | Height (cm) | Height relative to control |
|---|---|---|
| Control | 115 ± 4 (16) | |
| GmSMT1 | | |
| Line 6 | 114 ± 4 (16) | −1% |
| Line 51 | 109 ± 4 (10) | −6% |
| AtSMT2 | | |
| Line 6 | 94 ± 4 (10) | −18% (*) |
| Line 8 | 95 ± 3 (10) | −17% (*) |

## Discussion

Our results are consistent with a situation in GmSMT1 transformants where activity of the first methylation step of cycloartenol is raised, and that sterol precursors at the expense of cholesterol synthesis are channelled into the 24-methylated and 24-ethylated forms. The finding that this has caused an increase in the relative level of 24-methylcholesterol as compared with sitosterol, suggests that SMT2 activity is limiting in these transformants. The results are in agreement with data presented by Bouvier-Navé et al. (1998) who showed in a yeast experimental system that cycloartenol was the preferred substrate for NtSMT1, and that its enzymatic activity with 24-methylene-lophenol as a substrate was insignificant. Furthermore, the finding that the total sterol content was not affected in the transformants indicates that SMT1 does not have a rate-regulatory function in the biosynthesis of sterols in the tobacco leaf under normal conditions.

With respect to the AtSMT2 plants, the observed sterol profile with a lower contribution of both cholesterol and 24-methyl cholesterol, is in agreement with kinetic data from AtSMT2 expressed in yeast, which

showed that the enzyme is able to methylate cycloartenol as well as 24-methylene lophenol, albeit with a $V_{max}/K_m$ ratio only 6% relative to 24-methylene lophenol (Bouvier-Navé et al. 1997). Interestingly, the reduction in cholesterol levels was similar in GmSMT1 and AtSMT2 transformants. One possible explanation is that SMT2, which in the yeast membrane background exhibited poor activity with cycloartenol as substrate, is able to methylate this compound more efficiently in planta. Other possible explanations are effects due to metabolic fluxes or feedback regulation. The observation that stigmasterol levels in tobacco were unaffected by transformation with a 35S-AtSMT2 construct (Schaller et al. 1998) was here confirmed, and extended to SMT1 transformants. This suggests that the conversion of sitosterol to stigmasterol is a specifically regulated step in sterol biosynthesis.

Taken together, the results presented here for the GmSMT1 transformants suggest that the reduction in plant height in AtSMT2 transformants is due to the lower level of 24-methyl cholesterol, rather than to the decreased cholesterol or the increased sitosterol level per se. Recently, arabidopsis dwarf mutants have been identified which are impaired in their synthesis of 24-methyl cholesterol, a precursor of brassinosteroids (Klahre et al. 1998; Choe et al. 1999), and it is thus tempting to speculate that the reduced growth of AtSMT2 transformants is associated with alterations in brassinosteroid synthesis. However, brassinosteroid metabolism has been little investigated in tobacco, and a relationship between decreased growth and a possibly lowered brassinosteroid level in the AtSMT2 plants must await an analysis of brassinosteroid metabolism and its effect on plant growth in wild-type tobacco plants.

*In conclusion*, we have shown that overexpression of a SMT type-1 cDNA in tobacco results in decreased levels of cholesterol and increased levels of 24-alkylated sterols without significant effects on total leaf sterol content or plant growth and development.

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**Note added in proof.** During the revision process of this paper, similar results were published by Schaeffer A, Bouvier-Navé P, Benveniste P, Schaller H (2000) Lipids 35: 263–269