Sitosterol-β-glucoside as Primer for Cellulose Synthesis in Plants

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18. Transverse slices of hippocampi from Sprague-Dawley rats (18 to 30 days old) were obtained using standard methods (*30*). Slices were initially maintained in artificial cerebrospinal fluid (ACSF) containing a high concentration of sucrose: 87 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 75 mM sucrose, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7 mM $MgSO_4$, and 1 mM NaH_2PO_4. This medium was ice-cold for cutting, warmed to 35°C for 30 min immediately after, and then cooled to room temperature for a further 30 min. The ACSF was then switched to one that contained 119 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 1 mM NaH_2PO_4. All ACSF was equilibrated with 95% O_2 and 5% CO_2. The slices were then transferred to the experimental chamber, mounted on an upright microscope, where they were continuously superfused with normal ACSF (2 to 3.5 ml/min) at room temperature. Field excitatory postsynaptic potentials (fEPSPs) were recorded with extracellular electrodes filled with 1 M NaCl (2 to 4 megohms). D-2-Amino-5-phosphonovaleric acid (10 μM) was present throughout all LTP experiments. To elicit mossy fiber responses, we placed bipolar tungsten electrodes in the granule cell layer of the dentate gyrus. Frequency of stimulation was 0.05 Hz. Tetanic stimulation was 125 pulses, 25 Hz throughout the study. The group II metabotropic glutamate receptor agonist DCG-IV (1 to 2 μM) was applied at the end of each experiment to verify that the signal was generated by mossy fiber synapses. Patch-clamp recordings from CA3 pyramidal and dentate granule cells were obtained with either the whole-cell or perforated patch configuration. Cells were visualized using Nomarski-type differential interference contrast imaging with infrared illumination. The pipette solution (pH 7.4, 280 mOsm) contained 130 mM K-gluconate, 5 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, and 0.3 mM Na_2–adenosine triphosphate. For perforated patch recording, the tip was filled with a solution containing 120 mM KCl, 8 mM NaCl, 10 mM Hepes, 5 mM $CaCl_2$, 3 mM $MgCl_2$, and 5 mM QX-314Cl (RBI). The pipette was backfilled with this solution supplemented with amphotericin B (0.6 mg/ml). For perforated patch recordings, seals were attained in external solution that was nominally Ca-free. Borosilicate glass pipettes were pulled to tip resistances between 2 and 10 megohms. Access resistances were continuously monitored. Data were collected and analyzed using Igor Pro software. Synaptic responses were filtered at 2 to 20 kHz and digitized at 20 to 100 kHz. DK-AH269 was a gift from Boehringer Ingelheim. All measurements are given as means ± SE. Statistical significance was tested with Student's *t* test.
19. ZD7288 (50 μM) applied for 20 min caused a modest but consistent and stable reduction in synaptic transmission as measured by field potentials (~30%). This effect appeared to be due to a presynaptic action, because (i) a similar reduction of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–mediated excitatory postsynaptic currents was seen with whole-cell recording in which postsynaptic I_h was blocked by intracellular application of QX-314Cl (64 ± 9%, $n = 7$), (ii) the NMDA-mediated excitatory postsynaptic currents were similarly depressed (55 ± 14%, $n = 3$), and (iii) ZD7288 caused a small but significant increase in paired pulse facilitation (118 ± 5%, $n = 6$, $P < 0.05$), which correlates with a decrease in presynaptic release.
20. CsCl (1 mM) produced a >300% increase in fEPSP amplitude ($n = 6$). Once stability had been reached after ~60 min, tetanic stimulation or forskolin addition failed to further enhance the fEPSP ($n = 3$ each). Given the direct effect of Cs on the fEPSP, presumably unrelated to I_h, this blockade is difficult to interpret.
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Sitosterol-β-glucoside as Primer for Cellulose Synthesis in Plants

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Cellulose synthesis in plants requires β-1,4-glucan chain initiation, elongation, and termination. The process of chain elongation is likely to be distinct from the process of chain initiation. We demonstrate that a CesA glucosyltransferase initiates glucan polymerization by using sitosterol-β-glucoside (SG) as primer. Cotton fiber membranes synthesize sitosterol-cellodextrins (SCDs) from SG and uridine 5′-diphosphate–glucose (UDP-Glc) under conditions that also favor cellulose synthesis. The cellulase encoded by the *Korrigan* (*Kor*) gene, required for cellulose synthesis in plants, may function to cleave SG from the growing polymer chain.

Cellulose (β-1,4-glucan) microfibrils provide strength and flexibility to plant tissues and are also of great importance to wood, paper, textile, and chemical industries. Genetic evidence implicates plant *CesA* genes, homologous to bacterial cellulose synthases, and the *Korrigan* (*Kor*) gene, a membrane-associated cellulase, in cellulose synthesis (*1*–*5*).

Herbicides that disrupt cellulose synthesis include 2,6-dichlorobenzonitrile (DCB), which acts in a way that is not understood (*6*), and isoxaben, which may interact directly or indirectly with certain CesA proteins (*7*). Another herbicide, thiatriazene-based CGA 325'625, inhibits synthesis of crystalline cellulose and causes accumulation of a noncrystalline cellulose, which, upon treatment with cellulase, releases CesA protein (*8*) and a small amount of sitosterol linked to glucose (*9*). Because hydrophobic glycosides can function as primers for other glycosyltransferases (*10*–*14*), sitosterol-glucoside (SG) may serve as a primer for glucan chain elongation, an idea supported by observations that it is synthesized on the inner face of plant plasma membranes (*15*), where cellulose synthesis occurs. An enzyme, UDP-Glc:sterol glucosyltransferase (SGT), which is responsible for synthesis of sterol-β-glucosides, has been found associated with plasma membranes in various plants, including cotton fibers (*16*). Diglucosyl and triglucosylsterols of unknown function have also been isolated from rice bran (*17*).

When cotton fiber membranes are incubated with ^{14}C-labeled UDP-Glc in Tris buffer (*18*), two major compounds soluble in chloroform:methanol (C:M) are synthesized that migrate on thin-layer chromatography (TLC) as SG and SG acylated with palmitic acid (ASG) (Fig. 1A, lane 1). The glucose is in terminal linkage to sterol, >95% of which is sitosterol (*19*). Replacing Tris with Mops

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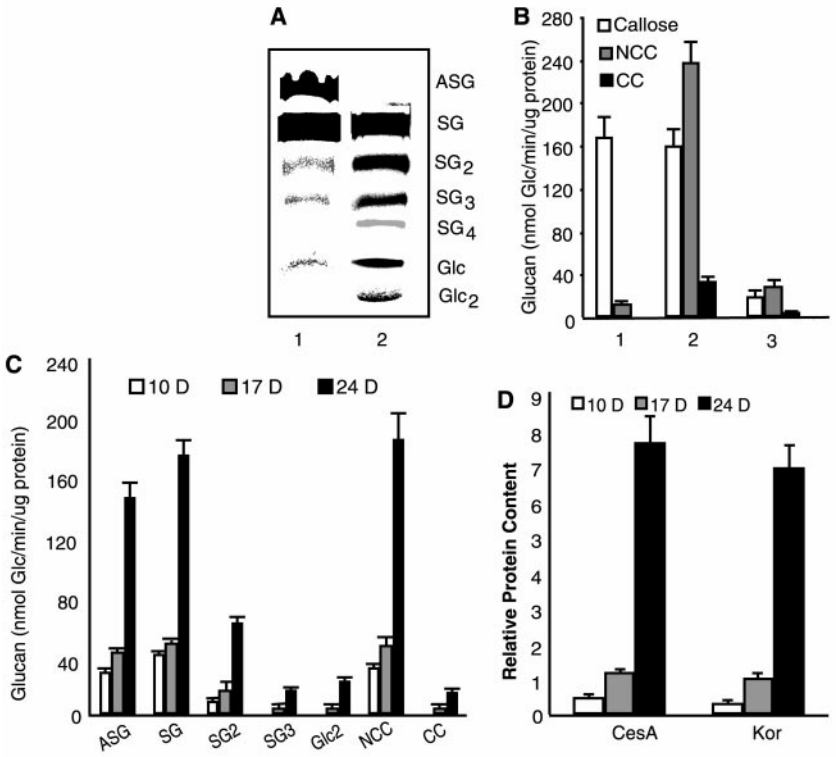

Fig. 1. Synthesis of SG, SCDs, and glucans by using cotton fiber membranes. CC, crystalline cellulose defined as glucan remaining insoluble after treatment with acetic-nitric reagent (*28*); NCC, noncrystalline cellulose defined as 4-linked glucan not resistant to acetic-nitric reagent. (**A**) Radioautogram of TLC separations of sterol derivatives synthesized from UDP-[^{14}C]Glc (*18*). Lane 1, buffered with Tris-HCl; lane 2, buffered with NaMops; Glc_2 refers to cellobiose. (**B**) Synthesis of glucans by fiber membranes. Callose is glucan in β-1,3 linkage (*18*). Lane 1, buffered with Tris; lane 2, buffered with Mops; lane 3, buffered with Mops and $CaCl_2$ in reaction replaced with 5 mM EGTA. (**C**) Synthesis of SG, SCDs, and glucan as a function of age of fibers used to harvest membranes (200 μg per reaction). 10 D, stage of primary-wall cellulose synthesis; 24 D, stage of maximal cellulose synthesis; 17 D, transition stage from primary- to secondary-wall synthesis. (**D**) Level of CesA and Kor proteins in fiber membranes (10 μg membrane protein per lane) as a function of fiber age; detection by Western blotting with antibodies against GhCesA-1 and the Kor proteins (*8*).

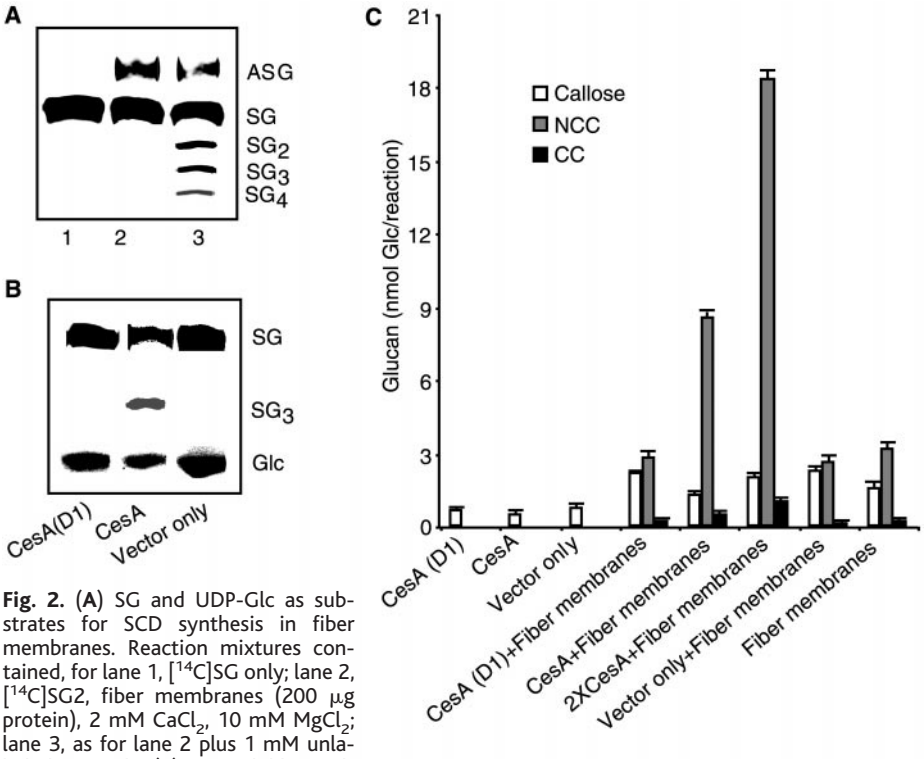

Fig. 2. (**A**) SG and UDP-Glc as substrates for SCD synthesis in fiber membranes. Reaction mixtures contained, for lane 1, [^{14}C]SG only; lane 2, [^{14}C]SG2, fiber membranes (200 μg protein), 2 mM $CaCl_2$, 10 mM $MgCl_2$; lane 3, as for lane 2 plus 1 mM unlabeled UDP-Glc. (**B**) C:M soluble products synthesized by yeast membranes with the use of [^{14}C]SG and UDP-Glc as substrates. See (*20*) for details of yeast expression and (*18*) for assay conditions using Na-Mops, $MgCl_2$, cellobiose, and $CaCl_2$ as effectors. Yeast and/or fiber membranes (200 μg membrane protein for each) were used per assay. Lanes: Membranes from yeast expressing D1 (Asp312Ala) mutant form of *GhCesA-1* (lane 1); lane 2, expressing wild-type *GhCesA-1*; lane 3, transformed with vector only. (**C**) Yeast membranes containing expressed *GhCesA-1* enhance synthesis of cellulose when incubated with fiber membranes. 2X CesA indicates that twice as many of the yeast membranes expressing *GhCesA-1* were added to reaction.

buffer leads to additional synthesis of the sterol cellodextrins (SCDs) S-cellobiose (SG2), S-cellotriose (SG3), and S-cellotetraose (SG4) [Fig. 1A, lane 2, and (*20*)]. Similarly, use of Mops favors synthesis of noncrystalline and crystalline cellulose over callose (β-1,3-glucan), and Ca^{2+} is required for synthesis of all glucans (Fig. 2B). Synthesis of SG, SCDs, and cellulose is highest in membranes isolated from fibers at the stage of active secondary-wall cellulose synthesis (Fig. 1C), when CesA and Korrigan protein levels are also highest (Fig. 1D).

Supplying a combination of [^{14}C]SG with unlabeled UDP-Glc to fiber membranes results in conversion of some of the SG to SCDs (Fig. 2A), indicating that a glycosyltransferase can use UDP-Glc to form SCDs from SG. Addition of [^{14}C]SCDs to membranes results in a labeled glucan product, supporting a role for SG as a primer [Web table 1 and (*20*)]. Kinetic studies also support a model in which SG is elongated to SCDs and subsequently to cellulose [Web fig. 1 and (*20*)].

To isolate the functions of CesA, an epitope-tagged cotton *GhCesA-1* cDNA was expressed in yeast (*20*). Yeast membranes contain some endogenous β-glucosidase activity, but only those expressing wild-type GhCesA-1 are capable of synthesizing SG3 from SG (Fig. 2B). Yeast with vector only or expressing a form of GhCesA-1 in which the critical Asp^{312} (D1) residue at the active site (*1*) is mutated to Ala, do not carry out this reaction. No cellulose synthesis was detected

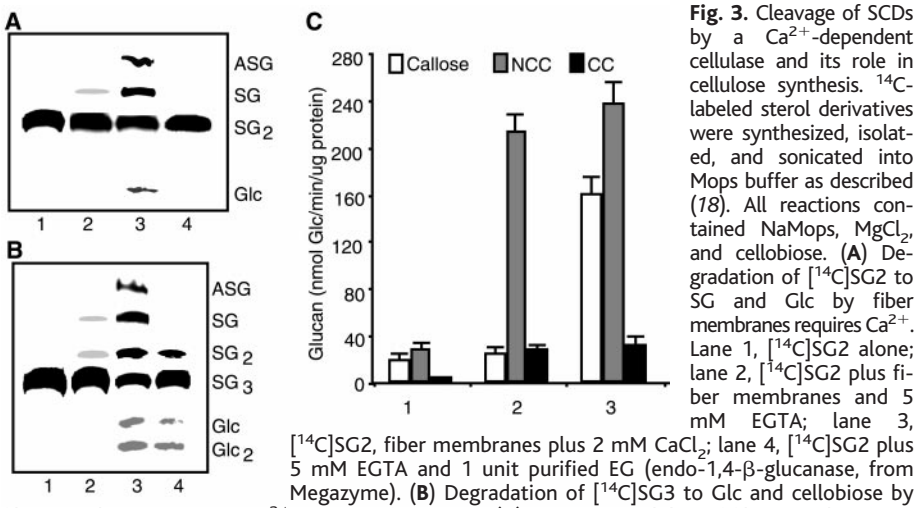

Fig. 3. Cleavage of SCDs by a Ca^{2+}-dependent cellulase and its role in cellulose synthesis. ^{14}C-labeled sterol derivatives were synthesized, isolated, and sonicated into Mops buffer as described (*18*). All reactions contained NaMops, $MgCl_2$, and cellobiose. (**A**) Degradation of [^{14}C]SG2 to SG and Glc by fiber membranes requires Ca^{2+}. Lane 1, [^{14}C]SG2 alone; lane 2, [^{14}C]SG2 plus fiber membranes and 5 mM EGTA; lane 3, [^{14}C]SG2, fiber membranes plus 2 mM $CaCl_2$; lane 4, [^{14}C]SG2 plus 5 mM EGTA and 1 unit purified EG (endo-1,4-β-glucanase, from Megazyme). (**B**) Degradation of [^{14}C]SG3 to Glc and cellobiose by fiber membranes requires Ca^{2+}. Lanes as for Fig. 3A. (**C**) EG restores ability of fiber membranes to synthesize cellulose from UDP-[^{14}C]Glc in the absence of Ca^{2+}. Lane 1, 5 mM EGTA; lane 2, 5 mM EGTA, 1 unit EG; lane 3, 2 mM $CaCl_2$.

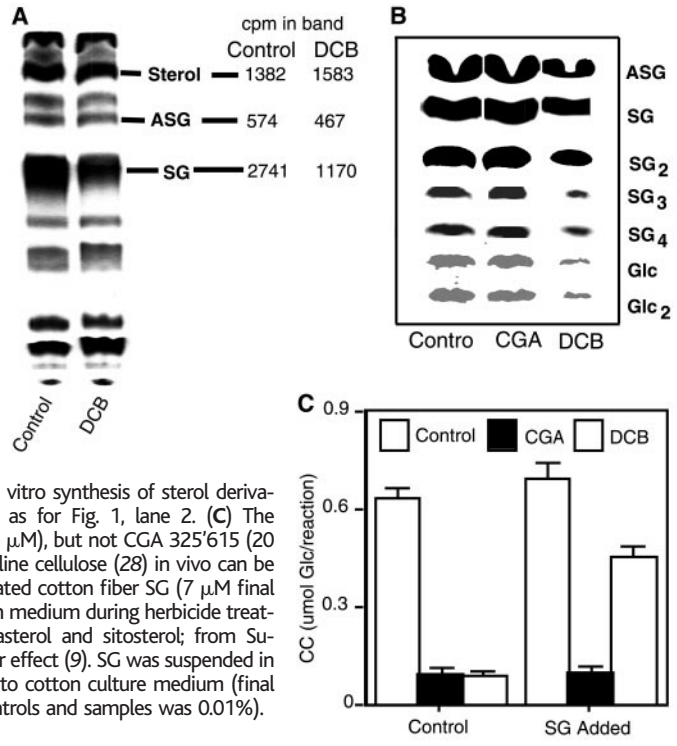

Fig. 4. DCB affects synthesis of SG, SCDs, and cellulose. (**A**) Synthesis of SG in vivo is reduced in cultured cotton fibers incubated with U-[^{14}C]Glc, as described (*8*), with or without 25 μM DCB. Material soluble in C:M was extracted from membranes, chromatographed, and subjected to radioautography (*18*). Amounts of the extract having equal counts per minute of radiolabel were loaded per lane. (**B**) Effects of in vivo herbicide treatments of fibers on capacity of membranes for in vitro synthesis of sterol derivatives. In vitro assays done as for Fig. 1, lane 2. (**C**) The inhibitory effect of DCB (25 μM), but not CGA 325'615 (20 nM), on synthesis of crystalline cellulose (*28*) in vivo can be reversed by addition of isolated cotton fiber SG (7 μM final concentration) to incubation medium during herbicide treatments. Free sterols (stigmasterol and sitosterol; from Supelco) did not show a similar effect (*9*). SG was suspended in 1% DMSO before addition to cotton culture medium (final DMSO concentration in controls and samples was 0.01%).

with the use of membranes expressing GhCesA-1, but such membranes do enhance cellulose synthesis when mixed with cotton fiber membranes (Fig. 2C). Such enhancement might occur through provision of substrate SG3 synthesized by transgene yeast membranes with the use of sterols from the cotton membranes or from provision of other factors necessary for complete chain elongation and termination.

The requirement for Ca^{2+} for in vitro cellulose synthesis (Fig. 1) might be explained by the fact that the Kor cellulase requires Ca^{2+} for activity (*20, 21*). A possible role for this enzyme might be to cleave sterol from the growing glucan chain to allow further chain elongation. Our reaction mixtures also produce cellobiose and glucose [Fig. 1 and (*20*)] that might come from cellulase-mediated cleavage of SCDs. In cotton membranes, SG2 and SG3 can be partially cleaved to SG and glucose or cellobiose by an endogenous Ca^{2+}-dependent activity, whereas a commercial endo-β-1,4-glucanase can only cleave SG3 (Fig. 3, A and B). When putatative Kor activity and cellulose synthesis are inhibited by EGTA, ability of cotton fiber membranes to synthesize cellulose can be restored by addition of very low levels of a Ca^{2+}-independent endo-β-1,4-glucanase (Fig. 3C). [Higher levels of this glucanase or longer incubation times lead also to degradation of the glucan product (*9*).]

An additional connection between the synthesis of SCDs and cellulose comes from the observation that DCB inhibits synthesis of SG in vivo (Fig. 4A). In vitro, SG and SCD synthesis is inhibited by DCB but not by CGA 325'615 (Fig. 4B). Inhibition by DCB is effective only if fibers are pretreated with DCB, not when it is added directly to isolated membranes; presumably some alteration in these activities must occur in vivo with DCB that cannot at present be mimicked in vitro. Addition of SG reverses the effect of DCB (but not CGA 325'615) on cellulose synthesis in vivo (Fig. 4C), suggesting that DCB inhibits cellulose synthesis via inhibition of SG synthesis.

We suggest a model [Web fig. 2 (*20*)] in which SG serves as a primer for β-1,4-glucan chain elongation catalyzed by CesA proteins. Kor likely functions to cleave SG from SCDs, which seemingly allows chain elongation to proceed more efficiently. Because the Glc moiety of SG is attached via its reducing end to the sterol, subsequent elongation should also proceed from the nonreducing end as predicted (*22*). It remains to be determined whether all cellulose-producing eucaryotes will be found to use SG as primer; procaryotes, lacking such sterols, either must not use a primer or must use some other compound. Because the model assumes that SG serves as primer in other plants besides cotton, it leads to certain predictions that can be tested. One is that mutants of *Korrigan* should accumulate SCDs that could be the "lipid-linked cellodextrins" that are reported to accumulate in *kor* mutants (*5*). Other predictions are that at least a partial cause of the severe phenotypes observed in sitosterol-reduced transgenic or mutant plants of *Arabidopsis* (*23, 24*) may be due to impairment of cellulose synthesis or that any mutants in genes encoding SGTs may lead to a similar reduction in cellulose synthesis. Finally, because yeast membranes expressing GhCesA-1 can elongate SG to SG3 but cannot synthesize cellulose except in the presence of added fiber membranes, factors other than just GhCesA-1 protein and SG must also be required for efficient chain elongation. In view of the suggestion that more than one nonidentical CesA may be required for cellulose synthesis (*7, 25, 26*), GhCesA-2 is a likely candidate. The challenge now is to identify the remaining factors required to reconstitute cellulose synthesis in vitro.

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18. Fibers (24 days after anthesis) from *Gossypium hirsutum* Coker 130 were ground in liquid N_2 and extracted at 4°C with 50 mM Na-Mops buffer (or 50 mM Tris-HCl as indicated), pH 7.5, 5 mM EDTA, 0.25 M sucrose, and protease inhibitor cocktail (Boehringer). Extracts were sequentially centrifuged at 2800*g* for 20 min, then at 100,000*g* for 1 hour, and the 100,000*g* membrane pellet was suspended in 50 mM Mops buffer, pH 7.5, containing protease inhibitors. Reaction mixtures containing 5 μM UDP-[^{14}C]Glc (0.5 μCi), 10 mM $MgCl_2$, 2 mM $CaCl_2$ (or 5 mM EGTA as indicated), 10 mM cellobiose, and 250 μg membrane protein in final volume of 0.25 ml were incubated for 1 hour at 37°C and terminated by heating at 100°C for 15 min. Chloroform:methanol (C:M, 2:1, 0.25 ml) was added with mixing, and the resulting upper phase was re-extracted five more times with C:M (2:1). C:M layers were pooled, chromatographed on Silica Gel G plates by using C:M:H_2O (65:25:4) as solvent, and subjected to radioautography. [^{14}C]Glucan products in the remaining interface and upper layer were either precipitated at 4°C in 70% ethanol and linkage patterns determined by gas-liquid chromatography (GLC) of permethylated products or by specific enzyme digestions (*8*).
19. SG was eluted from TLC plates in chloroform:methanol (C:M, 2:1), dried under N_2, and hydrolyzed with 2 M trifluoroacetic acid at 120°C for 1 hour, and extracted with C:M:H_2O (2:1:1). The H_2O/M phase was collected for sugar analysis by GLC (*27*), and the C:M phase was used for identification of the major sterol as sitosterol by retention time and mass spectrum with GLC/MS (DB-17 capillary column). A molecular ion (*m/z* 414) and prominent fragment ions diagnostic of sitosterol (*m/z* 255, 281, 303, 329, 381, 396) were observed. We also determined by GLC that the fatty acid acylated to SG to form ASG is palmitic acid.
20. Supplementary Web material is available on *Science* Online at www.sciencemag.org/cgi/content/full/295/5552/147/DC1.
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