Formation of glucosylceramide and sterol glucoside by a UDP-glucose-dependent glucosylceramide synthase from cotton expressed in *Pichia pastoris*

Inga Hillig[a], Martina Leipelt[a,1], Claudia Ott[a], Ulrich Zähringer[b], Dirk Warnecke[a,*], Ernst Heinz[a]

[a] *Institut für Allgemeine Botanik, University of Hamburg, Ohnhorststr. 18, 22609 Hamburg, Germany*
[b] *Research Center Borstel, Center for Medicine and Biosciences, Parkallee 22, 23845 Borstel, Germany*

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Abstract In plants, glucosylceramide (GlcCer) biosynthesis is poorly understood. Previous investigations suggested that sterol glucoside (SG) acts as the actual glucose donor for the plant GlcCer synthase (GCS). We addressed this question by generating a *Pichia pastoris* double mutant devoid of GlcCer and SG. This mutant was used for heterologous expression of the plant GCS. The activity of the GCS resulted in the accumulation of GlcCer and, surprisingly, a small proportion of SG. The synthesis of GlcCer in the transformed double mutant shows that the GCS is SG-independent, while the detection of SG suggests that in addition to the sterol glucosyltransferase, also the GCS may contribute in planta to SG biosynthesis.

Key words: Cerebroside; Glucosylceramide synthase; Glycosphingolipid; Sterol glucoside; *Pichia pastoris*

1. Introduction

Glucosylceramides (GlcCer) are constitutively synthesized membrane lipids in plants, animals, and fungi. In plants, these glycolipids have been detected mainly in plasma membranes (PMs) but they also occur in preparations of vacuolar, Golgi and endoplasmic reticulum (ER) membranes [1]. GlcCer is suggested to play a role in the transport of membrane proteins and lipids in mammalian cells [2–4]. In addition, synthesis and degradation of GlcCer are believed to contribute to the level of ceramide which is regarded as a second messenger involved in many cellular response reactions [5–7]. In contrast, GlcCer functions in plants are still unknown. It has been suggested that changes in GlcCer content and molecular species composition are involved in the response to various stresses [8,9]. In animals, the conversion of ceramide to GlcCer is catalyzed by a uridine diphosphate (UDP)-glucose-dependent GlcCer synthase (GCS, E.C. 2.4.1.80, UDP-glucose:ceramide glucosyltransferase) [10]. The activity of this enzyme was first determined in 1968 [10] and since then different enzymatic assays have been developed [11–13]. The animal GCS is localized on the cytosolic face of the Golgi membranes [14–16]. The cloning of a cDNA encoding the human GCS opened the way for subsequent studies involving methods of reverse genetics [17].

Compared to this, very little is known about the final step in GlcCer biosynthesis and the function of GlcCer in plant cells. As indicated by the presence of GlcCer in plants, also these organisms are expected to express GCS activity. But in vitro, this activity is very difficult to measure, and only a very few studies report the determination of GCS activity in microsomal membrane fractions from plant tissues [18,19]. These in vitro GCS assays were usually carried out with UDP-glucose as glucosyl donor, but ceramide glucosylation reached at most 10% of the sterol glucosyltransferase activity which is readily measured under these conditions. Experiments with microsomal membrane preparations from bean hypocotyls and radiolabeled sterol glucoside (SG) as glucosyl donor were interpreted as demonstrating the existence of a UDP-glucose-independent GCS activity in plants [18]. According to these data, the plant enzyme may have SG:ceramide glucosyltransferase activity.

Recently, we identified a plant cDNA, the deduced amino acid sequence of which showed a few but significant sequence similarities with mammalian GCS. Its heterologous expression in a GlcCer-deficient mutant of the yeast *Pichia pastoris* resulted in the accumulation of GlcCer [20]. These data show that plants possess a GCS activity homologous to the mammalian enzyme. However, these experiments did not identify the glucose donor for the plant GCS, since the expression host *P. pastoris* contains UDP-glucose as well as SG and both have to be considered as putative glucose donors.

The present work describes a novel approach to decide between the two alternative glucosyl donors. For this purpose we eliminated endogenous GlcCer and SG of *P. pastoris* by the generation of a double null mutant devoid of sterol glucosyltransferase and GCS activity. This glycolipid-free mutant was used to determine whether the heterologously expressed plant GCS induces GlcCer formation in the absence of SG.

*Corresponding author. Fax: (49)-40-42816 254.
E-mail address: warnecke@botanik.uni-hamburg.de (D. Warnecke).

[1] Present address: School of Biology, Georgia Institute of Technology, Atlanta, GA 30332-0230, USA.

Abbreviations: GCS, glucosylceramide synthase; GlcCer, glucosylceramide(s); PCR, polymerase chain reaction; SG, sterol glucoside(s); TLC, thin-layer chromatography

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2. Materials and methods

2.1. Media and growth of P. pastoris

P. pastoris cells were grown at 30°C in yeast synthetic dextrose medium complemented with uracil, adenine, and amino acids if required. For gene expression driven by the AOX1 promoter, 0.5% methanol was substituted for glucose.

2.2. Disruption of the P. pastoris sterol glucosyltransferase gene (UGT51B1)

To construct a *ugt51B1* (GenBank accession number AF091397) null mutant of the *P. pastoris* strain JC308 [21], a fragment of the coding region was replaced by the URA3 cassette. The URA3 cassette was amplified by polymerase chain reaction (PCR) with the vector pBLURA [21] as template and the following primers: *UraSanDIs* 5′-GGGTCCCCTGCAGAAATGGGGAGATAA-3′ and *UraBamHIas* 5′-GGATCCACTAGTGGTTTTCTGGGGGTA-3′.

The PCR fragment was cloned into pGEM®-T (Promega) resulting in pGURA. The URA cassette was excised by *San*DI and *Bam*HI from pGURA and cloned into a vector containing the *P. pastoris* sterol glucosyltransferase sequence. From this vector, pUGT51B1g [22], a 3507-bp fragment of the *UGT51B1* coding region was excised by *San*DI/*Bam*HI, which was replaced with the URA cassette resulting in pGURASGT. This construct comprised the URA cassette flanked by 0.8- and 2.4-kb regions homologous to the *UGT51B1* 5′ and 3′ non-coding regions. Digestion of pGURASGT by *Bsp*MI resulted in a linear vector which was used for *P. pastoris* transformation by electroporation. Transformants were screened for uracil prototrophy on minimal medium lacking uracil. The disruption of *UGT51B1* was confirmed by PCR (data not shown).

2.3. Disruption of the GCS gene in the sterol glucosyltransferase null mutant

To generate the double null mutant, the *ugt51B1* null mutant strain was transformed with the GCS gene disruption construct pML4 [20]. The replacement of the GCS gene (GenBank accession number AF364403) was confirmed by PCR (data not shown).

2.4. Expression of the plant GCS gene in P. pastoris

GCS genes from *Gossypium arboreum*, *Candida albicans*, *P. pastoris*, and *Homo sapiens* cloned into the vector pPIC3.5 (Invitrogen) (→pPGa, →pPCa, →pPPp, and →pPHs) [20] were expressed under the control of the strong AOX1 promoter in the double null mutant. Cells were grown at 30°C in YPD medium to an OD_{600} of 1.0–1.5, and transformed into minimal medium (without glucose) supplemented with 0.5% methanol to induce gene expression. Cells were harvested by centrifugation 15 h after induction and used for lipid analysis and in vitro enzyme assays.

2.5. GCS assay

P. pastoris cells were resuspended in buffer (100 mM Tris/HCl, pH 8.0; 15% glycerol) and disrupted by ultrasonication and vortexing with glass beads. Cell debris was removed by centrifugation at 13 000 rpm for 30 s. The supernatant fraction was centrifuged at 100 000 × *g* for 30 min at 4°C to obtain a membrane fraction. The assay mixture contained in a total volume of 100 μl: 80 μl of *P. pastoris* membrane fraction (0.5–1.0 mg of protein) and 500 000 dpm UDP-[U-^{14}C]glucose (specific activity 10 GBq/mmol, final concentration 8 μM). After incubating for 1 h at 30°C, the reaction was terminated by phase separation between 0.9 ml 0.45% NaCl solution and 4 ml chloroform/methanol 2:1. Half of each lipid extract was separated by thin-layer chromatography (TLC), while the other half was subjected to alkaline hydrolysis. For this purpose the lipids were dissolved in 1 ml $CHCl_3$ and incubated with an equal volume of 0.5 M sodium methoxide in methanol for 1 h. Phase separation was achieved by the addition of 1 ml $CHCl_3$ and 0.75 ml 0.45% NaCl solution and the extracted lipids were separated by TLC. The radioactivity on the silica gel plate was determined by radioscanning with a BAS-1000 BioImaging Analyser (Raytest, Straubenhardt, Germany).

2.6. Lipid extraction and analysis

Cells were harvested by centrifugation and kept for 10 min in boiling water. Lipid extraction was performed as described by Warnecke et al. [22]. The glycolipids were purified by silica gel column chromatography (Phenomenex Strata SI-1). After washing the column with 2 ml of $CHCl_3$, neutral lipids, glycolipids, and polar lipids were successively eluted with 8 ml of each $CHCl_3$, acetone/2-propanol 9:1, and methanol. The glycolipid fraction was further separated by preparative TLC developed with $CHCl_3$/methanol 85:15 and each glycolipid was acetylated for subsequent nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). When the amount of glycolipids was very low, the lipids were analyzed by gas chromatography and MS (GC-MS) without prior acetylation.

3. Results

3.1. Generation of a P. pastoris double null mutant lacking both SG and GlcCer

As outlined in the introduction, the construction of a double null mutant of *P. pastoris* devoid of SG and GlcCer was a prerequisite for our studies. The availability of new auxotrophic host strains of *P. pastoris* enables a more complex manipulation of its genome including the creation of double null mutants [21]. For this purpose the *P. pastoris* genes encoding the two relevant sterol and ceramide glucosyltransferases were sequentially disrupted. The disruption of the UDP-glucose:sterol glucosyltransferase gene resulted in the complete loss of SG in the mutant (Fig. 1, lane 2) with no effect on vegetative growth under normal culture conditions. This mutant was used as parental strain for the subsequent disruption of the GCS gene to generate the double null mutant Δ*ugt51B1*/Δ*gcs*. This double null mutant did not contain SG or GlcCer (Fig. 1, lane 3), but was still viable and grew like the parental strain in complex as well as in minimal medium supplemented with adenine and arginine. These results suggest that SG and GlcCer are not essential for normal growth of *P. pastoris*.

3.2. Expression of the GCS from cotton in the P. pastoris double null mutant resulted in GlcCer and SG biosynthesis

The double null mutant was used to express the recently

Table 1
Formulas of isolated GlcCer

TLC band	Molecular species	Systematic name
GlcCer 1	24:0 (2-OH)-t18:0	*N*-2′-hydroxytetracosanoyl-1-*O*-β-D-glucopyranosyl-4-hydroxysphinganine
	26:0 (2-OH)-t18:0	*N*-2′-hydroxyhexacosanoyl-1-*O*-β-D-glucopyranosyl-4-hydroxysphinganine
GlcCer 2	16:0 (2-OH)-18:1$^{\Delta 4}$	*N*-2′-hydroxypalmitoyl-1-*O*-β-D-glucopyranosylsphing-4-enine
	16:0 (2-OH)-18:2$^{\Delta 4,8}$	*N*-2′-hydroxypalmitoyl-1-*O*-β-D-glucopyranosylspinga-4,8-dienine
	16:0 (2-OH)-18:2$^{\Delta 4,8}$ 9m	*N*-2′-hydroxypalmitoyl-1-*O*-β-D-glucopyranosyl-9-methylsphinga-4,8-dienine
	18:0 (2-OH)-18:1$^{\Delta 4}$	*N*-2′-hydroxystearoyl-1-*O*-β-D-glucopyranosylsphing-4-enine
	18:0 (2-OH)-18:2$^{\Delta 4,8}$	*N*-2′-hydroxystearoyl-1-*O*-β-D-glucopyranosylsphinga-4,8-dienine
	18:0 (2-OH)-18:2$^{\Delta 4,8}$ 9m	*N*-2′-hydroxystearoyl-1-*O*-β-D-glucopyranosyl-9-methylsphinga-4,8-dienine
GlcCer 3	18:0-18:2$^{\Delta 4,8}$	*N*-2′-stearoyl-1-*O*-β-D-glucopyranosylsphinga-4,8-dienine

All glycolipids were recovered from the *P. pastoris* Δ*ugt51B1*/Δ*gcs* double null mutant expressing the cotton GCS. By TLC these GlcCer were resolved into three different groups (GlcCer 1–3 from bottom to top) from which GlcCer 1 and 2 are mixtures of various molecular species.

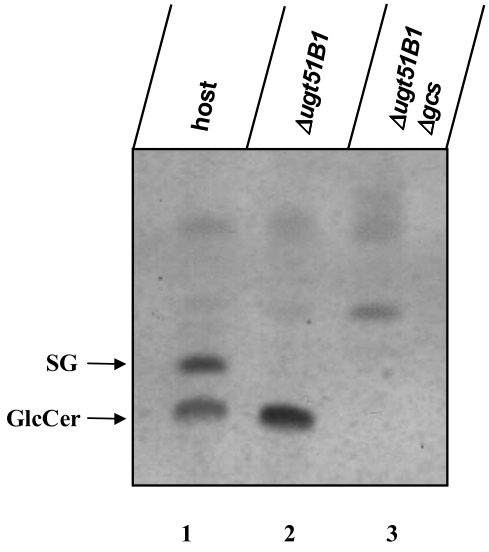

Fig. 1. Generation of a *P. pastoris* double null mutant lacking SG and GlcCer. Lipid extracts from *P. pastoris* cells were separated by TLC. Lane 1, the parental *P. pastoris* strain JC308 contained SG and GlcCer. Lane 2, disruption of the sterol glucosyltransferase gene (Δ*ugt51B1*) resulted in the complete loss of SG. Lane 3, additional disruption of the GCS gene (Δ*gcs*) in *P. pastoris* Δ*ugt51B1* caused the loss of GlcCer.

cloned GCS sequences from cotton (*G. arboreum*), *H. sapiens*, *C. albicans*, and *P. pastoris*. The activity of these different GCSs resulted in the accumulation of new glycolipids in the transformed yeast cells (Fig. 2). Expression of the cotton GCS resulted in the biosynthesis of four glycolipids with one being present in very low proportions (Fig. 2). In contrast, the expression of the GCS from *P. pastoris* resulted in the accumulation of only two components. The newly formed glycolipids were isolated, acetylated and subjected to structural analysis by MS and NMR spectroscopy. The majority of compounds were identified as GlcCer carrying a β-glucopyranosyl residue linked to the primary hydroxyl group of ceramides of different structure. Depending on the composition of the ceramide backbone regarding long-chain base and acyl substituent, the resulting glycolipids were resolved chromatographically into two or three groups containing different molecular species as detailed in Table 1. Besides GlcCer synthesis, the expression of the plant, but not of the fungal GCS resulted in the accumulation of SG, the structure of which was confirmed by mass spectroscopy. The GCSs from *C. albicans* and *H. sapiens* synthesized GlcCer which were not analyzed in detail. Like the GCS from *P. pastoris*, these enzymes do not form SG. We also performed an in vitro assay to determine the UDP-glucose-dependent activity of the overexpressed GCS from cotton, *H. sapiens*, and *P. pastoris* in membrane preparations obtained from the transformed yeasts. Incubation of the membrane fractions with UDP-[U-^{14}C]glucose resulted in the synthesis of labeled GlcCer which were identified by cochromatography with authentic GlcCer standard (Fig. 3, lanes 1, 3, and 5). These glycolipids withstood alkaline hydrolysis with sodium methoxide (Fig. 3, lanes 2, 4, and 6) which supports the assumption that they represent GlcCer. Acetylation of the radioactive compound recovered from the TLC plate and rechromatography resulted in a shift in R_f value due to increased hydrophobicity as also seen with unlabeled reference GlcCer (data not shown). Surprisingly, SG synthesis by the plant GCS could not be determined by the in vitro

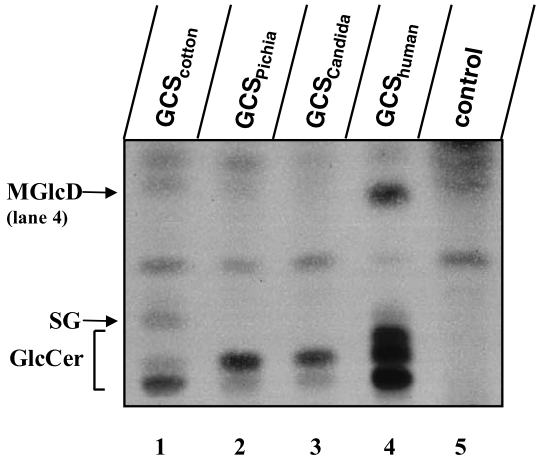

Fig. 2. Expression of the GCS from cotton in the *P. pastoris* Δ*ugt51B1*/Δ*gcs* double null mutant resulted in GlcCer and SG biosynthesis. Lane 1, the expression of the GCS from cotton resulted in the biosynthesis of three groups of GlcCer, from which GlcCer 3 is a minor component and only detectable after further purification (see Table 1). In addition, the plant GCS formed SG. Lanes 2 and 3, the expression of the GCSs from *P. pastoris* and *C. albicans* resulted both in the biosynthesis of two groups of GlcCer. Lane 4, the expression of the GCS from *H. sapiens* resulted in the biosynthesis of five groups of GlcCer plus MGlcD [20]. The GlcCer with the largest R_f value co-migrates with the SG standard, but further analysis showed that SG was not present in the lipid extract [20]. Lane 5, control cells transformed with the empty expression vector pPIC3.5 did not contain GlcCer or SG. The lipid band between MGlcD and SG was not identified, but due to its color after spraying with α-naphthol sulfuric acid it probably did not represent a glycolipid.

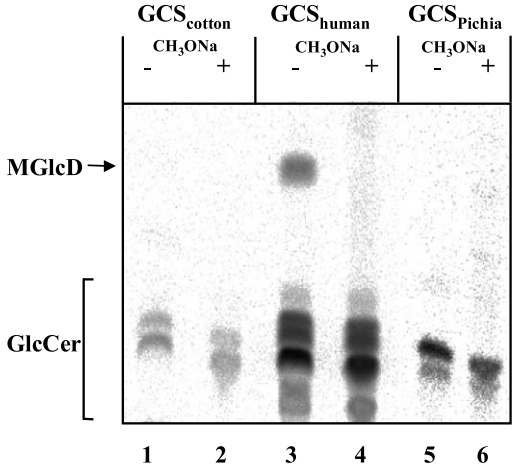

Fig. 3. In vitro activity of recombinant GCS enzymes from cotton, human, and *P. pastoris* expressed in the *P. pastoris* Δ*ugt51B1*/Δ*gcs* double null mutant. Membrane fractions from *P. pastoris* expressing the GCS from cotton, human, and *P. pastoris*, respectively, were used for in vitro enzyme assays containing radiolabeled UDP-glucose as substrate (see Section 2). The labeled lipophilic reaction products were separated by TLC with subsequent analysis by a BAS-1000 BioImaging Analyser. Aliquots of each reaction were subjected to alkaline hydrolysis before TLC. GCS from cotton (lane 1), *H. sapiens* (lane 3), and *P. pastoris* (lane 5) synthesized GlcCer which were resistant to alkaline hydrolysis (lanes 2, 4, and 6). In contrast, MGlcD produced by the human GCS was degraded by alkaline hydrolysis (lane 4). Control cells did not incorporate radioactivity from labeled UDP-glucose into glycolipids (not shown).

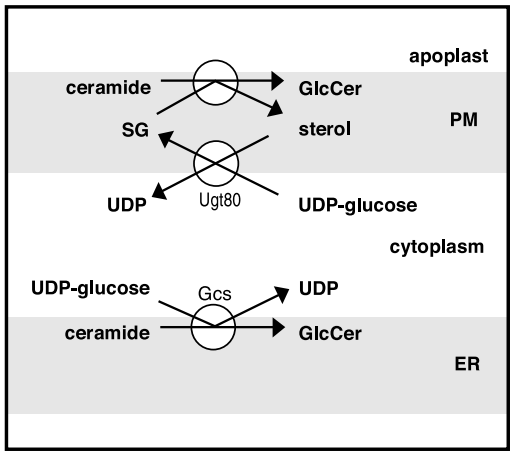

Fig. 4. Hypothetical model for alternatives in GlcCer formation in plants. The UDP-glucose-dependent GCS is a resident of the ER (unpublished results), probably facing the cytosolic side of the membrane similar to Golgi apparatus-located GCS from mammals. On the other hand, biochemical data suggest the existence of another GCS activity operating at the apoplastic side of the PM [23]. This enzyme has been suggested to use SG as glucosyl donor which is produced by the UDP-glucose:sterol glucosyltransferase Ugt80 localized in the PM and other membranes [25]. There may be a hypothetical flip-flop transfer of SG from the inner to the outer leaflet of the PM.

assay, which differs from the SG biosynthesis of the enzyme in the *Pichia* cells. The GCS from human additionally synthesized monoglucosyldiacylglycerol (MGlcD) [20], which was degraded by the alkaline hydrolysis (Fig. 3, lanes 3 and 4).

4. Discussion

The expressions of the GCS from cotton, *H. sapiens*, *C. albicans*, and *P. pastoris* in the SG- and GlcCer-free double null mutant of *P. pastoris* resulted in the accumulation of GlcCer. These data demonstrate that GCSs can produce GlcCer in the absence of SG, which is an unexpected result for the plant enzyme. An in vitro enzyme assay demonstrated that the fungal and plant GCSs accept UDP-glucose as sugar donor for GlcCer formation. Therefore, we assume that the yeast enzyme as well as the plant enzyme cloned from cotton do not require SG, but are UDP-glucose-dependent enzymes like the GCSs from mammals. This result confirms the expected substrate specificity of the yeast enzyme, but it does not support the hypothesis that plant GCS activity is SG-dependent [18]. Possible explanations for this discrepancy are discussed in the following.

The plant, the mammalian, and the fungal GCSs belong to the family 21 of nucleoside diphosphate (NDP)-sugar hexosyltransferases [20]. According to hydropathy plots, the enzyme encoded by the cDNA from cotton has a similar N-terminal transmembrane domain as other GCS proteins which are localized in Golgi membranes [14–16].

On the other hand, the products of GCS activity, i.e. the GlcCer, have been found also in the PM, the tonoplast and Golgi membranes of plant cells [1]. This situation requires lipid transport to deliver GlcCer to different membranes and/or another GCS activity with a different intracellular localization. Indeed, an SG-dependent GCS activity in wax bean has been ascribed to the apoplastic surface of the PM [23] (see also Fig. 4). Thus, in plant cells, different GCS activities could be present: preliminary experiments with a cotton GCS-green fluorescent protein (GFP) fusion protein expressed in onion cells point to an ER-concentrated enzyme (O. Kusmakow and E. Heinz, unpublished results). This GCS uses UDP-glucose as sugar donor, whereas others at the apoplastic side of the PM might use SG as glucosyl donor. In the genome of *Arabidopsis thaliana* we can find only a single GCS sequence with similarity to the cotton sequence. Therefore, we conclude that the corresponding enzyme uses UDP-glucose as donor. The SG-dependent GCS activity should be encoded by a sequence with no easily recognizable similarity to the 'normal' GCS. An insertion-tagged mutant of *A. thaliana* hit in the GCS sequence is being studied in our lab and will show whether GlcCer is still remaining, pointing to an alternative way of synthesis.

Interestingly, the UDP-glucose-dependent GCS from cotton is able to synthesize SG. This activity may be of particular importance, since SG has been suggested to be involved in the initiation of cellulose synthesis [24]. This hypothesis will be checked by the use of an insertion-tagged mutant of *A. thaliana* which is defective in the two or three sterol glucosyltransferase genes found in the *A. thaliana* genome. However, our present data suggest that it might be necessary to create a triple or quadruple mutant which is also impaired in the GCS activity to obtain a plant which is completely devoid of SG synthesis.

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