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Review

Recently discovered functions of glucosylceramides in plants and fungi

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Abstract. Glycosphingolipids are ubiquitous membrane lipids of eukaryotic organisms and a few bacteria. Whereas inositol-containing glycosphingolipids are restricted to plants and fungi, galactosylceramide occurs only in fungi and animals. In contrast, glucosylceramide is the unique glycosphingolipid which plants, fungi and animals have in common. However, there are specific differences in the structure of the ceramide backbone of glucosylceramides from these organisms. A comparison of the structural features and the biosynthesis of glucosylceramides from plants, fungi and animals will contribute to our understanding of their functions, which so far have been analysed mainly in animals. The availability of nearly all genes involved in the biosynthesis of glucosylceramides enables the specific manipulation of glycosphingolipid metabolism by techniques of forward and reverse genetics. Application of this approach to unicellular organisms like yeasts, multicellular filamentous fungi, as well as to complex organisms like plants will reveal common and different glucosylceramide functions in these organisms. These glycolipids play a role both in intracellular processes and in cell-to-cell interactions. These interactions may occur between cells of a multicellular organism or between cells of different species, as in host-pathogen interactions.

Key words. Pathogenicity; host-pathogen interaction; cerebroside; *Saccharomyces cerevisiae*; glucosylceramide synthase; UDP-glucose; ceramide glucosyltransferase: glycosyl phosphoryl inositol ceramide; glycosphingolipid.

Introduction

In recent years, glucosylceramide (GlcCer), the simplest member of the large group of glycosphingolipids, has attracted increasing attention, because unexpected and diverse functions are attributed to this sphingolipid. The success in recognizing these functions is mainly due to the fact that some genes controlling crucial steps in the biosynthesis of GlcCer have been cloned only recently from different phyla. These genes have been used to generate corresponding mutants which encouraged investigations to close the remaining gaps for complete coverage of the biosynthetic pathway up to GlcCer.

The aim of this review is to compile the structures of GlcCer from plants, fungi and animals and assemble identified and still hypothetical genes responsible for their biosynthesis. These data are required for reverse-genetics approaches to unravel functions of GlcCer headgroups and backbones. In addition, differences in GlcCer structures between plants, fungi and animals have turned out to be of particular importance for specific functions and GlcCer recognition in cell-cell interactions. Thus, structural peculiarities of GlcCer will be discussed in detail. Those readers mainly interested in an overview of the biological functions of GlcCer should go straight to the final section of this review which provides a comparison of GlcCer functions in plants, fungi and animals.

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During the past three decades, sphingolipids have been the subject of intensive research focussed on mammals and baker's yeast. This development was triggered by interest in the large number of different glycosyl headgroups of mammalian sphingolipids and the pathophysiology of their biosynthesis and degradation [1]. More recently, (E)-sphing-4-enine, ceramide, and sphingoid base phosphates have been found to act as signalling molecules [2–10]. Sphingolipids also mediate cell adhesion/recognition [11], serve as lipid moieties for glycosyl phosphatidyl inositol (GPI)-anchored proteins [12], and play a role in intracellular vesicle transport [13]. Sphingolipids which carry a glycosyl headgroup are typical membrane lipids of eukaryotic cells, although a few bacteria also contain glycosphingolipids [14]. Two main glycosphingolipid classes have been identified in eukaryotic cells. Ceramides linked to glycosyl moieties via inositol phosphate are called glycosyl inositol phosphoryl ceramides (GIPCs) and occur as free membrane lipids and as membrane anchors of covalently bound proteins (GIPC-anchors). The latter are also known as GPI-anchors, a term which combines lipid anchors containing either ceramide or diacylglycerol [15]. The second class consists of (mono)hexosylceramides (cerebrosides) with mainly β-D-glucose or β-D-galactose as substituents of the C-1 hydroxy group of the ceramide. These (mono) hexosylceramides also serve as precursors for a large variety of higher glycosylated sphingolipid species.

Since GIPCs only occur in plants and fungi, and galactosylceramide is restricted to fungi and animals, GlcCer represents the sole glycosphingolipid which is common to all eukaryotes including plants, fungi and animals. GlcCer plays a central role in mammalian sphingolipid metabolism, since it represents a biosynthetic intermediate for the formation of over 300 different complex glycosphingolipids [16, 17] and may contribute to the control of the level of ceramide [5, 8].

In many studies, the yeast *Saccharomyces cerevisiae* has served as a model organism to study sphingolipid metabolism and functions due to the availability of its complete genomic sequence, mutant and suppressor strains and knowledge of most of the genes involved in sphingolipid metabolism [18–21]. However, this statement does not apply to the biosynthesis and functions of GlcCer which are absent in baker's yeast. In contrast, most fungal species investigated so far contain GlcCer [22] (table 1) and, accordingly, baker's yeast has to be considered an exception.

Although GlcCer structures of fungi and plants have been analysed in detail, data on their biological functions are limited. Taking *S. cerevisiae* as a model, generation of mutants of fungi and plants affecting all genes involved in GlcCer metabolism will promote the elucidation of glycosphingolipid functions. Many fungi are suitable for random gene disruption (insertional mutagenesis) as well as targeted gene disruption by homologous recombination [23–26]. Targeted gene inactivation has not been established as an efficient technique for plants [27, 28], but several collections of *Arabidopsis thaliana* mutants generated by random transposon or T-DNA insertion have been generated [29, 30]. Since the complete genomic sequence of *A. thaliana* is available [31] and the sequencing of genomic regions flanking the inserted T-DNA is currently in progress for some mutant collections, mutants impaired in sphingolipid metabolism can easily be iden-

Table 1. Glycosylceramides in fungi.

Fungus	Sphingolipid	References
Acremonium chrysogenum	GlcCer	22
Amanita muscaria	GlcCer	85
Amanita rubescens	GlcCer	85
Aspergillus fumigatus	**GlcCer**, Δ3	71, 228
	GalCer, Δ3	71, 228
Aspergillus nidulans	**GlcCer**	223
Aspergillus niger	**GalCer**, Δ3	61, 229
	GlcCer, Δ3	229
Aspergillus oryzae	GlcCer	82
Aspergillus versicolor	**GlcCer**	228
Candida albicans	**GlcCer**	22, 64
Candida deformans	GlcCer	230
Candida utilis	GalCer	65, 66
Cryptococcus spp.	**GlcCer**	229
Fusarium solani	**GlcCer**, Δ3	231
Fusicoccum amygdali	**GlcCer**, Δ3	232
Ganoderma lucidum	**GlcCer**	216
Hansenula anomala	GlcCer	233
Histoplasma capsulatum	**GlcCer**, Δ3	67
Hypsizigus marmoreus	**GlcCer**	84
Kluyveromyces lactis	**GlcCer**	79
Lentinus edodes	**GlcCer**	81
Magnaporthe grisea	**GlcCer**, Δ3	210
	GalGlcCer*	77
Neurospora crassa	$Gal_3GlcCer$*	70
Pachybasium	**GlcCer**, Δ3	234
Paracoccidioides brasiliensis	**GlcCer**, Δ3	71
Pichia pastoris	**GlcCer**	22, 78
Polyporus ellisii	**GlcCer**	235
Pseudoallescheria boydii	**ClcCer**	227
Rhynchosporium secalis	**GlcCer**	22
Saccharomyces cerevisiae	GalCer	65, 66
Saccharomyces kluyveri	**GlcCer***	79
Schizophyllum commune	**GlcCer**	80, 214
Sordaria macrospora	GlcCer	22
Sporothrix schenckii	**GlcCer**, Δ3	75
	GalCer, Δ3	75
Termitomyces albuminosus	GlcCer	236
Metridium senile (sea anemone)	**GlcCer**	83

GlcCer, glucosylceramide; GalCer, galactosylceramide. Bold lettering indicates that the ceramide backbones of the respective glycosylceramides were confirmed to consist of C_{16} or C_{18} hydroxy fatty acids and (4E,8E)-9-methylsphinga-4,8-dienine (see fig. 2). Glycosylceramides containing a Δ3-desaturated fatty acid are marked by Δ3.

* At least portions of these glycosylceramides contain a 4-hydroxysphinganine/α-hydroxy-VLCFA-backbone which is typical for GIPCs but unusual for glycosylceramides.

tified in databases and may be supplied by the institutions which generated the collections [32–35] [see also the following web pages: http://signal.salk.edu/tabout.html; http://www.tmri.org/pages/collaborations/garlic-files/GarlicDescription.html; http://www.mpiz-koeln.mpg.de/GABI-Kat/]. These techniques are complemented by antisense gene inactivation [36–38].

In the following we will describe the structures of GlcCers from different phyla, and will include seemingly minor structural details. An example for the unexpected relevance of such details will be presented when discussing fungal sphingoid bases.

Plants possess a large variety of different GlcCers while most fungi share a consensus GlcCer structure

Plants ubiquitously contain GlcCers as minor components accounting for less than 5 mol% of the total lipid. However, highly purified plasma membrane (PM) and tonoplast preparations showed that these lipids comprise from 6 to 27 mol% of membrane lipids (table 2). Since GlcCers are predominantly present in the outer monolayer of the PM, they represent a major component of plant lipids facing the apoplast [39]. Structural features of

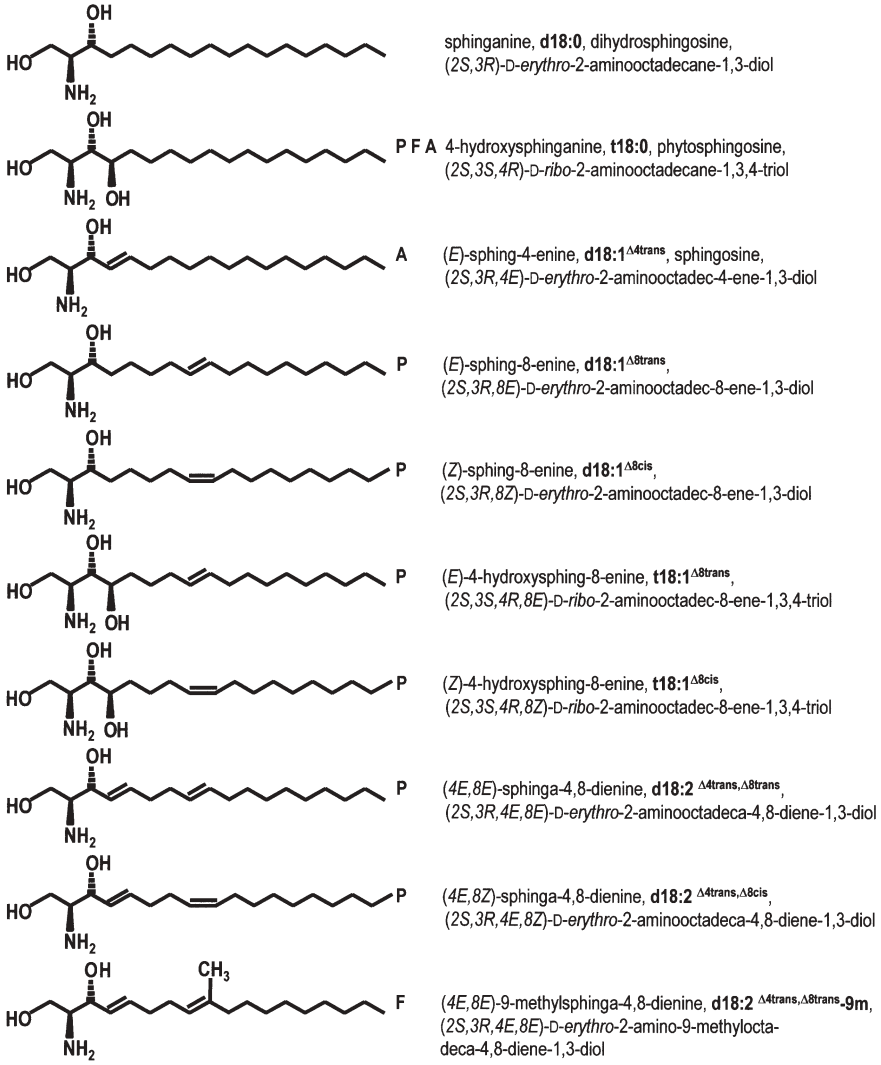

Figure 1. Common sphingoid bases from plants, fungi and animals. Trivial names, shorthand designations and systematic names according to IUPAC recommendations 1997 [247] (http://www. chem.qmul.ac.uk/iupac/misc/glylp.html) are given for each sphingoid base. All naturally occurring dihydroxy sphingoid bases are in D-erythro and all trihydroxy sphingoid bases are in D-ribo configuration. Only common C_{18} sphingoid bases are depicted in the figure, but sphingoid bases of different chain length also occur in minor amounts. P, F or A indicate that the sphingoid base is typical for plants, fungi or animals, respectively. 4-Hydroxysphinganine is a minor sphingoid base in mammals, but occurs in ceramides from human skin in considerable amounts [248]. In plant and fungal glycosylceramides, 4-hydroxysphinganine has been detected only in minute amounts, whereas it represents the dominant sphingoid base in plant and fungal GIPC and GIPC-anchors [12, 93, 94].

Table 2. Lipid composition of purified membranes from plants (in mol% of total lipid).

Source	Membrane	Remark	GlcCer	SG	ASG	FS	PL	Ref.
Arabidopsis thaliana	PM	leaves	7	5	3	38	47	42
	PM	leaves	4	5	3	31	57	42
Avena fatua, wild oat	PM	leaves, HS	22	10	n.d.	27	30	237
	PM	leaves, HR	26	11	n.d.	30	29	237
Avena sativa, spring oat	PM	leaves	27	6	27	8	29	51
spring oat	PM	leaves, CA	24	8	22	8	37	51
winter oat	PM	leaves	30	4	25	10	29	51
winter oat	PM	leaves, CA	23	6	22	9	40	51
	PM	roots	9	<3	3	9	80	57
	PM	roots, DA	5	<3	3	14	75	57
	PM	roots	10	10	5	25	50	238
	PM	coleoptiles	26	7	6	20	42	238
	TP	protoplasts	21	11	13	n.d.	30	165
Hordeum vulgare, barley	PM	leaves	23	n.d.	n.d.	28	39	239
	TP	protoplasts	23	7	8	4	42	166
	ER	roots	8	2	1	5	73	164
	TG	roots	14	3	2	10	63	164
	PM	roots	8	7	6	30	45	164
Kalanchoe digremontiana	TP	leaves	12	6	3	21	47	166
Mesembryanthemum	TP	leaves, C3	9	10	2	21	39	166
crystallinum	TP	leaves, CAM	11	8	3	27	43	166
	TP	leaves, C3	9	10	2	21	39	166
	TP	protoplasts	9	11	11	15	43	166
Secale cereale, rye	PM	leaves	16	6	3	38	37	51
	PM	leaves, CA	10	4	1	41	43	51
	PM	leaves	16	15	4	33	32	203
	PM	leaves, CA	7	6	1	44	42	203
Solanum tuberosum	PM	leaves	7	9	32	4	46	240
	PM	leaves, CA	5	12	26	8	47	240
Solanum commersonii	PM	leaves	6	7	32	2	48	240
	PM	leaves, CA	5	5	31	5	51	240
Vigna radiata, mung bean	PM	E	7	2	2	40	49	167
	TP	E	17	2	7	18	51	167
Triticum aestivum, wheat	PM	L, CT	14	7	8	34	37	204
	PM	L, CT, CA	10	3	4	39	43	204
	PM	L, CT, E	12	7	6	37	39	204
	PM	L, CT, E, CA	10	6	2	42	40	204
	PM	L, CS, E,	15	4	6	28	47	204
	PM	L, CS, E, CA	12	4	4	33	47	204
	PM	roots, CT	14	20	11	22	32	204
	PM	R, Al-R,	7	9	5	28	46	241
	PM	R, Al-R, 20 µM Al	8	10	6	22	49	241
	PM	R, Al-S,	6	10	5	33	43	241
	PM	R, Al-S, 20 µM Al	4	11	4	32	43	241
Triticum durum	PM	roots	9	5	6	28	52	242, 243
	PM	roots, 0 µM Cu	9	4	3	25	59	242, 243
	PM	roots, 50 µM Cu	10	2	2	22	63	242, 243
Zea mays, corn	PM	roots	7	6	3	41	44	48

Al-R, aluminium-resistant cultivar; Al-S, aluminium-sensitive cultivar; C3, photosynthetic mechanism producing phosphoglycerate as the first stable intermediate in CO_2 fixation; CA, cold acclimated; CAM, crassulacean acid metabolism; CS, cold-sensitive wheat variety Talent; CT, cold-tolerant wheat variety Roughrider; DA, drought acclimated; E, etiolated; HR, herbicide resistant; HS, herbicide susceptible; L, leaves; n.d., not determined; PM, plasma membrane; R, roots; TG, tonoplast plus Golgi; TP, tonoplast; ER, endoplasmic reticulum.

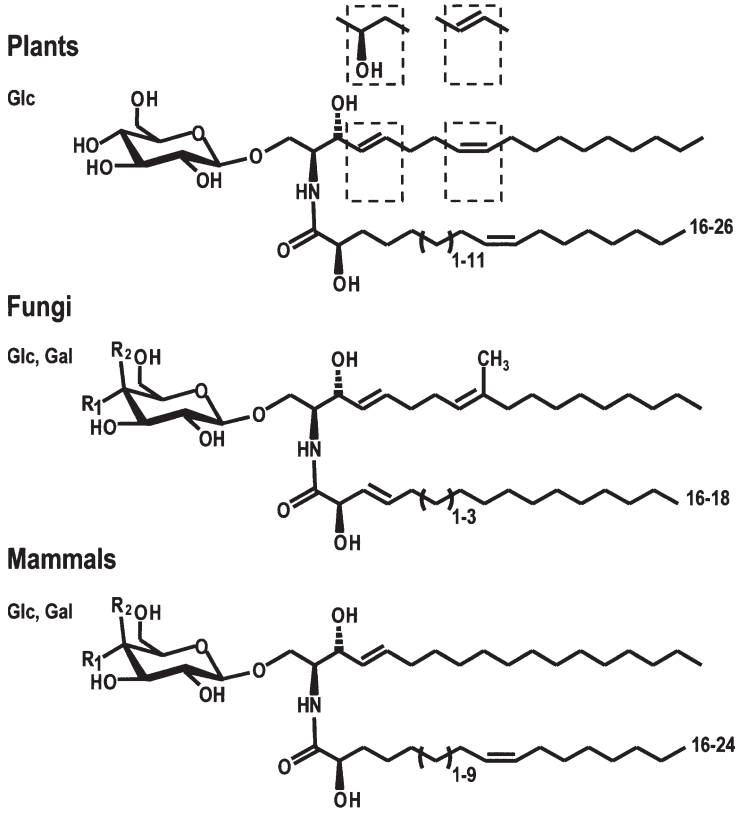

Figure 2. Glycosylceramides from plants, fungi and mammals. The ceramide backbones of glucosylceramides from plants (at the top) show great variety. Their sphingoid bases carry three different possible modifications: hydroxylation or trans desaturation at C-4, and cis or trans desaturation at C-8, the combination of which results in seven frequent plant sphingoid bases (see also fig. 1). These sphingoid bases are linked to more than ten different α-hydroxy fatty acids varying in chain length and n-9 desaturation. In contrast to plants, glucosyl- and galactosylceramides from fungi (middle) show a characteristic fungal consensus structure with only a few structural variations. The sphingoid base (4E,8E)-9-methylsphinga-4,8-dienine is linked to α-hydroxy C_{16} or C_{18} fatty acids which in some fungi are trans-desaturated at C-3 resulting in (E)-2-hydroxyhexadec-3-enoic and (E)-2-hydroxyoctadec-3-enoic acid. 4-Hydroxysphinganine and VLCFA have been detected in fungal glycosylceramides only rarely (see text). Mammalian glucosyl- and galactosylceramides (bottom) contain the characteristic sphingoid base (E)-sphing-4-enine, which is rare in plants and fungi, linked to an α-hydroxy or non-hydroxy fatty acid of 16–24 carbons. Longer ω-hydroxylated fatty acids with up to 34 carbons occur in ceramides of the skin (not shown). These ceramides also contain the sphingoid base 4-hydroxysphinganine (not shown). Fatty acid n-9 desaturation of sphingolipids in mammals and plants is restricted to the amide-linked VLCFA. Monosaccharide may be either glucosyl (Glc) (R_1 = OH, R_2 = H) or galactosyl (Gal) residues (R_1 = H, R_2 = OH).

GlcCers from plants have been analysed in detail particularly by Y. Fujino, M. Ohnishi and S. Ito [reviewed in refs 40, 41]. These data are summarized here and complemented by recent studies. The ceramide backbones of GlcCer from plants are very variable, being composed of six different C_{18} sphingoid bases and more than ten different α-hydroxy fatty acids. Sphingoid bases from plant GlcCers are derivatives of the C_{18} sphingoid base D-erythro-sphinganine (d18:0, fig. 1) showing three different possible modifications: hydroxylation or trans desaturation at C-4, and cis or trans desaturation at C-8. Combination of these modifications results in eight sphingoid bases derived from sphinganine given in figure 1. Sphinganine and (E)-sphing-4-enine are found in low amounts in plants, whereas the latter is the predominant sphingoid base in animal sphingolipids. 4-Hydroxysphinganine (t18:0) has been detected in only minute amounts in GlcCers of many plants, but it has been identified as a major component in *A. thaliana* in a single study [42]. Thus, both Δ8 cis/trans isomers of each d18:1$^{\Delta 8}$, t18:1$^{\Delta 8}$, and d18:2$^{\Delta 4,\Delta 8}$ are the predominating sphingoid bases in plant cerebrosides [43–46]. Fatty acids of plant GlcCers are almost exclusively α-hydroxylated and vary in chain length from C_{14} to C_{26} with C_{16}, C_{20}, C_{22} and C_{24} fatty acid as major components [40, 47, 48]. The enrichment of sphingolipids with such fatty acids in rafts points to the relevance of this structural motif, as is also evident from a sphingolipid suppressor mutant which will be discussed below. Saturated fatty acids predominate in plant GlcCer, whereas n-9 monounsaturated very long chain fatty acids (VLCFAs) from C_{22} to C_{26} occur in low amounts [49]. However, these unsaturated fatty acids are abundant in some cereals [50]. Studies on the molecular species composition of plant GlcCer show that almost all hypothetical

combinations of sphingoid bases and fatty acids occur in nature. More than 20 different GlcCer species, of which up to 12 each comprise more than 1 mol% of the total GlcCer, have been determined in some plant species [42, 51–58]. The sugar moiety found in plant glycosylceramides is usually D-glucopyranose in β linkage to the C-1 hydroxy group of the sphingoid base. However, mannose can also be attached to the ceramide backbone, and higher homologues with up to four sugars as a linear chain were isolated from cereals [41].

Fungi contain two types of glycosphingolipids: glycosylceramides, with mainly glucose or galactose moieties, and GIPC [59]. The core structure of GIPC consists of ceramide linked at C-1 to inositol-1-phosphate. The inositol moiety of GIPC is usually substituted at the hydroxy group at C-2 (rarely at C-6) by α-D-mannose which often carries another inositol phosphate or various glycan chains [59, 60]. Since GIPCs have been isolated from several fungi, and all fungi investigated so far contain GlcCer or GalCer (table 1), most species likely contain both types of glycosphingolipids. This statement has been confirmed for several fungi such as *Aspergillus niger* [61, 62], *Candida albicans* [63, 64], *Candida utilis* [65, 66], *Histoplasma capsulatum* [67–69], *Neurospora crassa* [70], *Paracoccidioides brasiliensis* [71, 72], *S. cerevisiae* [65, 66, 73, 74] and *Sporothrix schenkii* [60, 75, 76]. However, detailed structural data of the ceramide backbones of both types of glycosphingolipids are available for only five fungal species (tables 1, 3).

Interestingly, these two types of glycosphingolipids have different ceramide backbones. While C_{16} or C_{18} hydroxy fatty acids linked to (4E,8E)-9-methylsphinga-4,8-dienine (d18:2$^{\Delta 4trans,\Delta 8trans}$-9m) are exclusively precursors of GlcCer synthesis, very long chain C_{24} and C_{26} hydroxy fatty acids bound to 4-hydroxysphinganine are restricted to the synthesis of GIPC [22, 59, 71]. Some studies report the presence of 4-hydroxysphinganine and C_{24} hydroxy fatty acids in GlcCer, demonstrating that the ceramide glucosyltransferase is able to accept both ceramide backbones as substrates [70, 77–79]. Apart from these exceptional examples, many fungi obviously maintain two separate ceramide pools for the biosynthesis of GlcCer and GIPC. This separation could be achieved either by different ceramide selectivities of GlcCer synthase (GCS) and inositol phosphoryl ceramide synthase or by restricting access of the enzymes to cytologically separate ceramide pools. The mechanism of this ceramide discrimination remains to be elucidated.

In comparison with plants, GlcCer backbones of fungi are less varied and show a characteristic fungal consensus structure with only a few structural variations (fig. 2). As mentioned above, the sphingoid base of 19 carbons was identified as (4E,8E)-9-methylsphinga-4,8-dienine. *S. cerevisiae* synthesizes additional C_{16} and C_{20} sphingoid bases, particularly under stress conditions [19], which may also occur in other fungi, but they do not seem to be incorporated into GlcCer [22]. The fatty acids found in GlcCer were 2-hydroxypalmitic and 2-hydroxystearic acid which may carry a trans-double bond at C-3 in some fungi resulting in (E)-2-hydroxyhexadec-3-enoic and (*E*)-2-hydroxyoctadec-3-enoic acid (table 1). As mentioned above, VLCFAs have been found only in exceptional examples [70, 77–79] or in minor amounts [80, 81]. Whether these unusual GlcCer species are 'unwanted' products of a leaky separation of the two ceramide pools or whether they fulfill specific functions is not clear. The sugar headgroup of fungal glycosylceramides is mainly β-D-glucose, but some fungi also contain β-D-galactose (table 1). There are a few reports of more complex glycosylceramides with additional galactose residues attached to a GlcCer core structure [70, 77]. In summary, fungal GlcCers mainly consist of a β-D-glucose attached to C-1 of the methyl-branched, diunsaturated sphingoid base (4E,8E)-9-methylsphinga-4,8-dienine which is N-acylated with a long-chain hydroxy fatty acid. Only three structural variations occur: a galactosyl instead of a glucosyl headgroup, C_{16} and C_{18} fatty acids and a trans desaturation at C-3 of the fatty acid. From these data, one can conclude that fungi synthesize a characteristic consensus GlcCer structure differing from the diversity of plant GlcCers. However, this conclusion should not lead to the preconceived idea that some minor fungal glycosylceramide species mentioned above do not fulfil a biological function.

GlcCers in fungi account for 0.5–3 mol% of total lipid [22, 82–86], but there are no data on the GlcCer content

Table 3. Ceramide backbone composition of GIPCs from fungi.

Fungus	Ceramide backbone	References
Candida albicans	[24:0(h)-26:0(h)]- [t16:0-t18:0]	63, 244
Histoplasma capsulatum	24:0(h)-t18:0	68
Lentinus edodes	24:0h-t18:0	245
Paracoccidioides brasiliensis	24:0 (few h18-25)-t18:0	72
Sporothrix schenkii	24:0(hh)- [t18:0-t20:0]	60, 246

Only those fungi are considered from which ceramide structures of glucosylceramides have been determined (table 1).
22:0 h, α-hydroxy fatty acid of 22 carbons without double bond; (h), both fatty acids with and without α-hydroxy group have been found; t18:0, saturated trihydroxy sphingoid base (4-hydroxysphinganine).

of particular membranes. Lipid analyses of purified fungal subcellular membranes have been performed only with *S. cerevisiae* [87–89]. Sphingolipids in the PM of this yeast represent mainly GIPC and constitute about 30% of total phospholipids [90]. Since *S. cerevisiae* contains only minute amounts of galactosylceramide and no GlcCer at all [65, 66], a calculation of the quantitative proportions of the two above-mentioned different ceramide pools used for GlcCer and GIPC biosynthesis is not possible with this organism. Such studies have to be performed with fungi which contain both types of glycosphingolipids. Preliminary examinations of the sphingoid base composition of alkali-hydrolysed cells revealed that *Pichia pastoris* contains 10–100 times more 4-hydroxysphinganine than (4E,8E)-9-methylsphinga-4,8-dienine, pointing to a predominance of GIPC compared to GlcCer [unpublished results]. In this context, we should point out that comprehensive quantitative sphingolipid analyses of whole cells and particular membranes are urgently needed to complete our understanding of membrane composition and assembly. Studies in this direction are hampered by the fact that extraction of GIPC from the cells with organic solvents, and their purification are rather difficult compared to GlcCer. Besides glycerolipids, free sterols and sterol glycosides, such studies should cover the different sphingolipids such as free ceramides, glycosylceramides, free GIPC, and GIPC-anchors. A growing number of GPI-anchored proteins have been identified [91] and their lipid anchors have been analysed qualitatively but not quantitatively so far. They contain either diacylglycerol or ceramide esterified with inositol phosphate [15]. The inositol carries a glycan chain which is linked via an ethanolamine phosphate to the C terminus of the protein. This core structure, namely protein-CO-NH-$(CH_2)_2$-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6myo-inositol-PO_4-ceramide/diacylglycerol, is conserved, with some variations for all eukaryotic organisms [12, 92]. In both plants and fungi, the ceramide consists of an α-hydroxy or non-hydroxy VLCFA (plants C_{22} and C_{24}, baker's yeast C_{26}) and 4-hydroxysphinganine which may be desaturated at C-8 in plants resulting in 4-hydroxysphing-8-enine [12, 93, 94] (animal GPI-anchors contain only diacylglycerol). Thus, ceramide backbones of GIPC-anchors resemble the structures of free GIPC from fungi. However, the relative proportions and absolute amounts of GlcCer, free GIPC and GIPC-anchors in fungal and plant membranes need to be determined.

Mammalian glucosyl and galactosyl ceramides typically contain (E)-sphing-4-enine, which is rare in plants and fungi, linked to a fatty acid of 16–24 carbons (fig. 2). However, in many tissues, variants of the ceramide backbone have been found such as α-hydroxylated fatty acids or ω-hydroxylated fatty acids with up to 34 carbons [95–97]. (E)-sphing-4-enine may be replaced by 4-hydroxysphinganine.

In conclusion, the most striking differences between eukaryotic GlcCer are the occurrence of C_{16}-C_{18} fatty acids as well as VLCFA in GlcCer from plants and animals, whereas fungi separate two sphingolipid pools with C_{16}-C_{18} fatty acids found in GlcCer, and VLCFA found exclusively in GIPC and GIPC-anchors. In addition, the fungal sphingoid base of GlcCer is characterized by a methylation at C-9.

Most of the genes responsible for GlcCer biosynthesis have been cloned

We will now discuss recent progress in the cloning and identification of genes involved in GlcCer synthesis, with an emphasis on one representative species from each kingdom: *S. cerevisiae, A. thaliana* and *Homo sapiens* (fig. 3). From baker's yeast, most genes of sphingolipid metabolism have already been functionally characterized, and this applies also to many mammalian genes. The availability of these genes facilitates the generation of mutants impaired in sphingolipid metabolism either by targeted gene deletion or by random mutagenesis followed by screening techniques to study the functions of different members of this lipid class [19–21, 98, 99]. In addition, many of the respective proteins need further biochemical characterization. GlcCer from plants and most fungi share structural features which are not present in GlcCer from mammals and GIPC from baker's yeast. Therefore, cloning and characterization of genes responsible for GlcCer biosynthesis and use of reverse-genetics techniques are also required for these organisms. In comparison with *S. cerevisiae*, the availability of mutant lines and screening tools for other fungi and plants is still in its infancy, but this situtation is improving rapidly. To encourage such a systematic approach for GlcCer, sequences from *A. thaliana* and *C. albicans* are included in the following compilation of cloned and putative genes of GlcCer biosynthesis. The complete genomic sequence of *A. thaliana* and seeds from several mutant collections are available (see above). *C. albicans* is the first GlcCer-containing fungus for which the complete genomic sequence is available in public databases [http://www-sequence.stanford.edu/group/candida]. The data from *C. albicans* presented here may also facilitate identification and cloning of respective target genes in other fungi which are suitable for examining general or specific questions of sphingolipid functions.

All genes mentioned below are listed in table 4.

Serine palmitoyltransferase (*LCB1, LCB2, TSC3*)

Sphingolipid biosynthesis starts with the condensation of palmitoyl-CoA and serine generating 3-ketosphinganine. The reaction is catalysed by serine palmitoyltransferase,

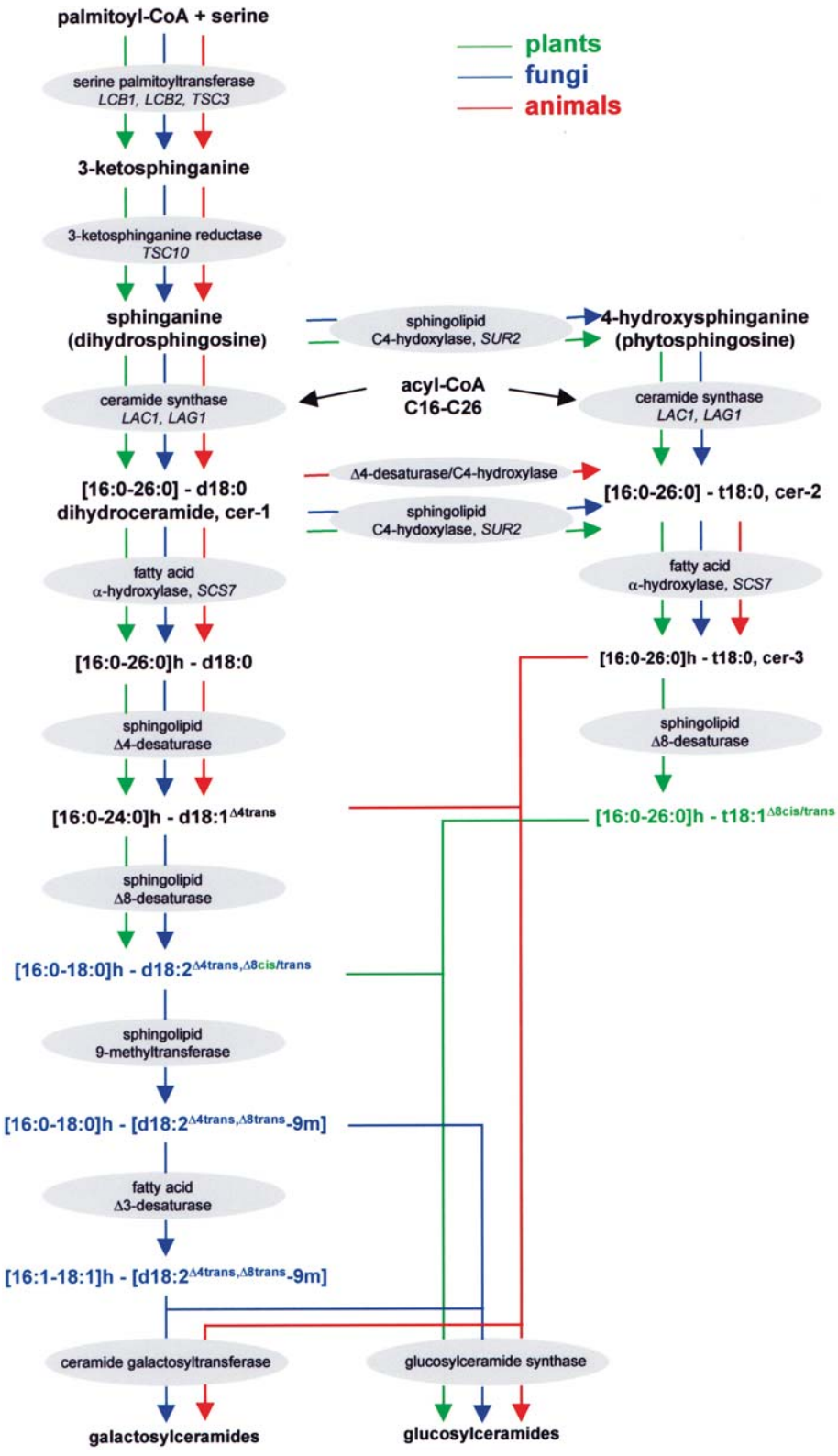

Figure 3. Overview of common features and differences in glycosylceramide synthesis of plants, fungi and animals. Metabolites are shown in bold lettering, enzymes and their genes stand out on a grey background. Genes which occur in *S. cerevisiae* are given in the preferred designations listed in the Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces). The sequence of some steps of glycosylceramide synthesis (in particular Δ4 and Δ8 sphingoid base desaturation) and the existence of the methyltransferase and the Δ3-desaturase are hypothetical. For further information on each step see the text. Biosynthetical branches such as the synthesis of sphingoid base phosphates, sphingomyeline, GIPC, GIPC-anchors and higher homologues of glycosylceramides are not depicted. VLCFAs introduced in ceramides from animals and fungi may be n-9 desaturated and those from mammalian skin contain up to 34 carbons (not shown). Some fungi synthesize GalGlcCer containing 4-hydroxysphinganine and VLCFA (not shown).

Table 4. Genes involved in glucosylceramide synthesis.

Gene	*S. cerevisiae*	*C. albicans*	*A. thaliana*	*H. sapiens*
Serine palmitoyltransferase	***LCB1* = YMR296C = M63674** ***LCB2* = YDR062W = M95669 = L33931**	orf 6.7816, contig 6-2502 orf 6.7982, contig 6-2502	AB063254 **AB046384** AB074928	**NM006415** **Y08686**
	***TSC3* = YBR085C-A = NP_116327**	not found	not found	not found
3-Ketosphinganine reductase	***TSC10* = YBR265W = NP_009824**	orf6.5112, contig 6-2388	NM_111481 NM_121925	XM_167461
Sphingolipid C-4-hydroxylase	***SUR2* = YDR297W = AAB64733**	orf6.4041, contig 6-2307	**AF361856** **AY070765**	not found
Ceramide synthase	***LAC1* = YKL008C** ***LAG1* = YHL003C**	orf6.8249, contig 6-2509 orf6.8514, contig 6-2513	AF198179 AF198180	NM_021267 AF177338[a]
Ceramidase	***YPC1* = YBR183W = AF191745** ***YDC1* = YPL087W = AF214455**	orf6.6941, contig 6-2477	BAB60897	**AF214454**[b]
Fatty acid α-hydroxylase	***SCS7* = YMR272C**	orf6.7517, contig 6-2495	**AY050326 = AF021804** AY058151	BC017049
Sphingolipid Δ4-desaturase	not found	**orf6.4414, contig6-2340**	AF220201	**AF466375** XM_058681[c]
Sphingolipid Δ8-desaturase	not found	orf6.548, contig 6.1607	**AJ224161** **NM_130183**	not found
Sphingolipid methyltransferase		unknown		
Glucosylceramide synthase	not found	**HSX11, contig 6-2503**	AF424585[d]	**D50840**
GlcCer galactosyltransferase	not found	not found	not found	**AF097159**
Ceramide galactosyltransferase	not found	not found	not found	**U30930**

Genes listed in the table are identified by their recommended names listed in the *Saccharomyces* genome database, http://genome-www.stanford.edu, if available and/or by GenBank nucleotide or protein accession numbers. The function of those genes depicted in bold letters have been verified experimentally. Genes depicted in plain letters has been identified by comparisons of their deduced amino acid sequences. For *A. thaliana* and *H. sapiens*, cDNA not genomic sequences are given.

[a] A murine homologue of the gene has been characterized experimentally [122].

[b] This mammalian ceramidase is homologous to *YPC1* from *S. cerevisiae*. Mammals contain several other ceramidases (not shown).

[c] A murine homologue has been identified as a bifunctional Δ4-desaturase/C-4-hydroxylase [116].

[d] A homologue from cotton has been characterized experimentally [78].

a membrane-bound enzyme localized on the cytosolic side of the endoplasmic reticulum (ER) of mammalian cells [100]. Detection of serine palmitoyltransferase activity in plant microsomes by in vitro enzyme assays points to a similar localization [101]. The enzyme consists of two essential subunits, Lcb1 and Lcb2 [102, 103]. Both genes have been cloned and functionally characterized from *S. cerevisiae* [104–106] and from mammals [107, 108]. An *LCB2* cDNA from *A. thaliana* has also been functionally expressed in *S. cerevisiae* [109]. Interestingly, *A. thaliana* contains a second hypothetical *LCB2* gene awaiting characterization, as does the putative *LCB1* gene. Hypothetical *LCB1* and *LCB2* genes from *C. albicans* have been identified due to their calculated amino acid sequence similarity to serine palmitoyltransferase from *S. cerevisiae*. A *TSC3* gene from *S. cerevisiae,* necessary for serine palmitoyltransferase activity at elevated temperatures (37 °C), is a peculiarity of this organism, since homologues have not been identified in any other species [110].

3-Ketosphinganine reductase (*TSC10*)

The second gene in sphingolipid biosynthesis, *TSC10*, encoding the membrane-bound enzyme 3-ketosphinganine reductase, is also essential for *S. cerevisiae* [111]. Homologues encoding putative 3-ketosphinganine reductases have been found in *C. albicans*, *A. thaliana* and *H. sapiens*. In mammalian cells, the enzyme is located on the cytosolic side of the ER [100].

Sphingolipid C-4-hydroxylase (*SUR2*)

S. cerevisiae is able to hydroxylate sphingolipids at the C-4 position of the sphingoid base. Whether the hydroxylase accepts sphinganine or ceramide or both substrates is not yet clear. This enzyme activity results in the formation of 4-hydroxysphinganine (phytosphingosine) or the corresponding ceramide. The hydroxylase gene *SUR2* has been cloned and deleted in *S. cerevisiae* and turned out to be non-essential [112, 113]. One of the few differences between the wild type and the *sur2*Δ mutant is the increase in the C_{20} to C_{18} sphingoid base ratio in the mutant [114]. To date, the functions of sphingoid base hydroxylation remain obscure, but some hints on its putative functions are reviewed by Dickson and Lester [115]. In *A. thaliana*, two sphingolipid C-4-hydroxylase genes have been identified and characterized [114]. *C. albicans* contains one putative *SUR2* gene. Mammals apparently do not possess similar sphingolipid C-4-hydroxylase genes, since the most closely related mammalian sequence to *SUR2* from *S. cerevisiae* is a C-4-methylsterol oxidase. However, some mammalian tissues contain hydroxylated sphingolipids which may be products of a bifunctional sphingolipid C-4-hydroxylase/Δ4-desaturase [116].

Ceramide synthase (*LAC1* and *LAG1*)

In *S. cerevisiae,* the sphingoid base is N-acylated by two similar and redundant acyl-CoA-dependent ceramide synthases, Lac1 and Lag1 [117, 118]. Deletion of both genes impairs sphingolipid biosynthesis, but is not lethal, which may be due to alternative acyl-CoA-independent ceramide synthesis by the reverse activity of two ceramidases, *YPC1* and *YDC1*, using free fatty acids as substrates [117, 118]. Two putative ceramide synthases have also been identified in *H. sapiens* [119–121], and a murine homologue of one of them probably represents a fumonisin-B1-resistant, stearoyl-CoA-specific ceramide synthase [122]. *A. thaliana* [123] and *C. albicans* each possess two putative ceramide synthases, but one of the genes from *C. albicans* shows only few similarities to Lac1 and Lag1 from *S. cerevisiae*. In baker's yeast, ceramide synthases obviously incorporate C_{26} fatty acids into ceramides, but not clear is whether their homologues from plants and fungi also accept C_{18} fatty acids found in GlcCer. Mammalian ceramide synthase activity is located at the cytosolic face of the ER [100, 122, 124].

Genes involved in VLCFA biosynthesis

Since GIPCs from plants and fungi and also GlcCers from plants and animals contain VLCFA, enzymes for fatty acid elongation are required to provide ceramide synthases with VLCFA-CoA. Recent investigations of this pathway have been discussed elswhere [21, 115, 125–131].

Ceramidase (*YPC1* and *YDC1*)

Two ceramidases preferring either N-acyl-4-hydroxysphinganine (phytoceramide) (Ypc1) or N-acyl-sphinganine (Ydc1) have been cloned from *S. cerevisiae* [132, 133]. These enzymes are probably involved in sphingolipid degradation but, interestingly, Ypc1 also shows acyl-CoA-independent reverse activity forming ceramide [132, 133]. A quadruple mutant of ceramide synthases and ceramide hydrolases (*lag1*Δ*lac1*Δ*ypc1*Δ*ydc1*Δ) does not synthesize any ceramides, but survives, probably because it carries a suppressor mutation leading to the synthesis of novel lipids [117]. Mammals contain acid, neutral and alkaline ceramidases localized in the lysosomes, the ER, the Golgi apparatus and mitochondria, respectively [134–137]. Putative ceramidases are also present in *A. thaliana* and *C. albicans*.

Fatty acid α-hydroxylase (*SCS7*)

Sphingolipids from fungi and plants usually contain α-hydroxylated fatty acids. A non-essential fatty acid hydroxylase gene has been cloned and characterized from *S. cerevisiae* [112, 138]. *A. thaliana* possesses two simi-

lar genes, one of which has been identified as a sphingolipid fatty acid hydroxylase [138]. Most mammalian sphingolipids contain non-hydroxy fatty acids except for a considerable proportion of galactosylceramide which contains α-hydroxy fatty acids. Putative hydroxylases from mammals and *C. albicans* require functional characterization. In *S. cerevisiae,* the hydroxylase encounters VLCFAs as substrates, but its homologues in other fungi may also accept C_{16} and C_{18} fatty acids. Not clear yet is whether the fungal hydroxylase accepts fatty acids of different chain lengths for both GlcCer and GIPC synthesis. Sphingolipid fatty acid α-hydroxylases probably do not use free or activated fatty acids, but rather prefer ceramides or complex sphingolipids as substrates [139]. The fact that plant ceramide synthase does not accept hydroxy fatty acids is consistent with the assumption that fatty acid hydroxylation occurs at the ceramide level [101]. In *S. cerevisiae*, α,β-dihydroxy fatty acids have been found in sphingolipids, raising the question whether the α-hydroxylase is also responsible for the β-hydroxylation. Alternatively, biosynthetic intermediates of the fatty acid elongation process containing a β-hydroxy group may be incorporated into ceramides and, subsequently, be subjected to α-hydroxylation [129, 130].

Sphingolipid Δ4-desaturase

Sphingolipids with unsaturated sphingoid base moieties have not been found in *S. cerevisiae* nor have sphingolipid desaturases been cloned from this organism. In contrast, other fungi as well as plants and animals synthesize sphingolipids with Δ4-trans-desaturated sphingoid bases. The reaction product of the desaturase, (E)-sphing-4-enine (sphingosine), is the major sphingoid base of mammalian sphingolipids. The Δ4-trans-desaturation is carried out at the cytosolic face of the ER at the level of the ceramide, whereas free sphinganine or GlcCer are not accepted as substrates of the desaturase [140, 141].

Sphingolipid Δ4-desaturase genes have recently been cloned and identified from *C. albicans*, *Drosophila melanogaster* (AF466379), *Mus musculus* (AF 466376 and AF 466377) and *H. sapiens* [116]. Heterologous expression of these genes/cDNAs in a *S. cerevisiae sur2*Δ mutant resulted in the biosynthesis of Δ4-desaturated sphingolipids. Mouse and human each contain two Δ4-desaturases, one of which turned out to be a bifunctional sphingolipid Δ4-desaturase/C-4-hydroxylase (mouse, AF466377; its human homologue XM_058681 has not yet been tested) [116]. This enzyme is probably responsible for the synthesis of 4-hydroxysphingolipids in mammals. Plant Δ4-desaturase-like sequences have been identified in *A. thaliana* and *Lycopersicon esculentum* (tomato, AF466378) but their functional expression in *S. cerevisiae* failed [J. Napier and P. Ternes, personal communication].

Interestingly, the biological relevance of Δ4-sphingolipid desaturase genes had been identified before the enzymatic function of their gene products was unravelled. The genes were known as *DES* and *MLD* in *D. melanogaster* and mammals, respectively [142–144]. A *des1* mutant of *D. melanogaster* is blocked at the G_2/M transition of meiosis in spermatogenesis. This defect points to a signalling function of Δ4-desaturated sphingolipids, but the Δ4-desaturated metabolite required for meiosis has not yet been identified.

Sphingolipid Δ8-desaturase

Like Δ4-desaturation, Δ8-desaturated sphingolipids also do not occur in baker's yeast, in contrast to many other fungi and plants. Interestingly, only trans isomers of Δ8-desaturated sphingolipids have been found in fungi, whereas a mixture of cis and trans isomers is typical for plants. cDNAs encoding sphingolipid Δ8-desaturases have been cloned from several plants [145–147]. Expression of these sequences in *S. cerevisiae* surprisingly revealed that they were responsible for the synthesis of both Δ8-cis- and Δ8-trans-double bonds. The plant sphingolipid Δ8-desaturases were the first stereo-unselective cis/trans desaturases found in nature. The enzymes tested required a C-4-hydroxylated substrate suggesting Δ8-desaturation to succeed the C-4-hydroxylation yielding 4-hydroxysphing-8-enine-containing sphingolipids. As mentioned above, non-hydroxylated sphing-8-enine is, however, present in plant GlcCers which may point to a second Δ8-desaturase activity in plants that is C-4-hydroxy independent and required for the synthesis of sphing-8-enine and sphinga-4,8-dienine. Indeed, a second Δ8-desaturase-like sequence has been identified in *A. thaliana* (NM_130183). Expression of this cDNA in the *S. cerevisiae sur2*Δ strain and culture of the cells with or without 4-hydroxysphinganine surprisingly revealed the C-4-hydroxy dependence of this enzyme also [P. Sperling and E. Heinz, unpublished data]. Thus, at present, the concurring substrate specificity of both enzymes expressed in *S. cerevisiae* is inconsistent with the existence of sphing-8-enines in plants, and the sequence of hydroxylation and desaturation of plant sphingoid bases remains to be elucidated.

Putative sphingolipid Δ8-desaturase genes have been identified in fungi like *C. albicans*, *Kluyveromyces lactis* (AB085690), and *Saccharomyces kluyveri* (AB085689). Expression of a corresponding gene from *P. pastoris* in *S. cerevisiae* resulted in the synthesis of trans-Δ8-desaturated sphingolipids [P. Sperling and E. Heinz, unpublished data]. This result is consistent with the occurrence of only trans-8 double bonds but not cis-Δ8 double bonds in GlcCer from fungi.

Sphingolipid methyltransferase

Methyl-branching at C-9 of sphingoid bases is a characteristic feature of fungi and a few other eukaryotes (table 1). Neither biochemical data on the methyltransferase nor sequence data on a methyltransferase gene are available. Since this methyl-branching may confer a particular function on the corresponding GlcCer, cloning and characterization of a sphingolipid methyltransferase are of particular importance. Sphingolipid C-9-methyl-branching represents a basic difference between sphingoid bases from fungi and from plants or animals, which may contribute to the interaction of pathogenic fungi with their host organisms (see below).

Fatty acid Δ3-trans-desaturase

Some fungal glycosylceramides contain an (E)-Δ3-desaturated fatty acid. This trans double bond has been found only in GlcCer and galactosylceramide from Euascomycetes but not in Hemiascomycetes or Basidiomycetes (table 1) [67]) The proportion of Δ3-desaturated to saturated fatty acids is higher in mycelium forms than in yeast forms of *P. brasiliensis* and *H. capsulatum*, suggesting involvement of this double bond in the yeast-mycelium transition of these pathogenic fungi [67, 71]. However, the biological functions of Δ3-fatty acid desaturation are still obscure. A Δ3-desaturase gene has not been cloned from any organism so far, and no candidate sequence has been identified in genome databases or expressed-sequence-tag libraries.

Fatty acid n-9 desaturation

n-9 cis-desaturated VLCFAs have been found in mammals and in some plants. Interestingly, this double bond occurs in many chilling/cold-resistant plant species such as rye, wheat, oat and *A. thaliana*, but not in sensitive plants [42, 50, 51], raising the question whether such sphingolipid unsaturation is involved in chilling/cold resistance. The formation of this double bond may be attributed to the plastidial Δ9-acyl-ACP desaturase. Its product, oleoyl-ACP (18:1-ACP), is hydrolysed, and free oleic acid exported from the plastid to the cytosol, activated to oleoyl-CoA and subjected to sequential steps of fatty acid elongation. This results in a series of n-9 monounsaturated VLCFAs of 22–26 carbons which could obviously serve as substrates for the plant ceramide synthases.

GCS

The first cDNA coding for a GCS (UDP-glucose:ceramide β-D-glucosyltransferase) was isolated from *H. sapiens* by an expression cloning technique using ceramide glucosyltransferase-deficient mouse melanoma cells [148]. The mammalian GCS contains a single putative transmembrane domain at the N terminus and a segment of mainly hydrophobic amino acids at the C terminus which may interact with the membrane. Biochemical studies showed that they are integral membrane proteins with their active site and the C terminus on the cytosolic face of the Golgi membrane [149–151]. The mammalian GCS belongs to family 21 of NDP-sugar hexosyltransferases [152] (P. M. Coutinho and B. Henrissat, Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). The same family has also been described as group 9 of the NRD2 glycosyltransferases which were grouped with respect to different types of nucleotide recognition domains [153]. This family shares few but significant similarities with the glycosyltransferase family 2 [152]. These similarities have been described as the D1,D2,D3,Q/RXXRW motif [154]. *S. cerevisiae* and *Schizosaccharomyces pombe* do not possess homologues of this protein family, consistent with the assumption that these yeasts do not synthesize GlcCer.

Recently, novel GCSs from other fungi and from plants have been identified by their functional expression in *S. cerevisiae* and *P. pastoris* [78]. These proteins show remarkably low overall sequence similarities to the mammalian GCS. The identities to the human glucosyltransferase are 16–21% for the proteins from the fungi *P. pastoris*, *C. albicans* and *Magnaporthe grisea* and only 9% for the protein from the plant *Gossypium arboreum* (cotton). All sequences share a few conserved amino acids and an N-terminal transmembrane domain. Thus, intracellular localization and membrane orientation of these ceramide gluocsyltransferases seem to be similar. A so far hypothetical 'mammalian-like' localization of the plant GCS at the cytoplasmic face of the Golgi apparatus is in contrast to biochemical data which hint at an orientation towards the apoplastic side of the PM [155]. Since UDP-glucose is absent in the apoplast, such a localization of a UDP-glucose-dependent GCS would not enable GlcCer synthesis. Unravelling these inconsistent data requires analysis of the substrate specificity of GCS and determination of its activity in different subcellular membranes. However, such an approach has for a long time been hampered by the lack of a simple in vitro enzyme assay for plant GCS. Whereas animal GCS was first identified in 1968 [156] and different enzymatic assays have been developed [157–159], activity of the plant enzyme was detected only recently [101, 160, 161].

In vitro synthesis of GlcCer by a microsomal membrane preparation from bean hypocotyls with radiolabelled sterol glucoside as a substrate unexpectedly demonstrated UDP-glucose-independent GCS activity [160]. The assumption that sterol glucoside serves as a glucose donor for glucosyltransferase reactions is paralleled by the novel finding that sterol glucosides act as primers for cellulose synthesis in plants [162, 163]. In an attempt to

check the sterol glucoside dependence of the cloned GCS from cotton, a sterol glucosyltransferase/GCS double-null mutant of the yeast *P. pastoris* was generated. This mutant, which was devoid of both sterol glucosides and GlcCer, was used for the expression of the cDNA encoding the ceramide glucosyltranferase from cotton leading to GlcCer synthesis [I. Hillig and E. Heinz, unpublished data]. This result demonstrates that the cotton GCS is sterol glucoside independent and probably uses UDP-glucose as a sugar donor. The enzyme can be assumed to be of Golgi origin, since GlcCers have been found not only in the PM, but also in the tonoplast and Golgi membranes of plants (table 2) [164–167]). Localization of GCS activity exclusively at the apoplastic site of the PM would require a transport of lipids from the PM to intracellular membranes which is common in mammals and yeasts but has not yet been shown for plants. Thus, there may be the possibility that plants possess two different GCS activities, a Golgi-located UDP-glucose-dependent enzyme and a PM-bound sterol glucoside-dependent activity. It is a matter of speculation whether the latter activity determined by Lynch and coworkers [160] may be attributed to the enzyme(s) responsible for the initiation of cellulose synthesis. Future studies will solve open questions on the sugar donors and location of the glucosyltransferases by making use of sterol glucosyltransferase and ceramide glucosyltransferase null mutants of *A. thaliana* and expression of green fluorescent protein fusions proteins.

Not only do the sugar donor(s) of the plant GCS have to be determined, but there is also little information on the substrate specificity of plant and fungal GCS towards the ceramide acceptor. Studies with the mammalian enzyme show that it accepts UDP-glucose and the natural ceramide N-acyl-D-erythro-sphinganine but not its L-erythro enantiomer or its L-threo diastereoisomer [168]. Overexpression of GCS from mammals, fungi and plants in *P. pastoris* resulted in the biosynthesis of many GlcCer species differing in the degree of sphingoid base decoration with double bonds, hydroxy groups and methyl side chains [78]. The molecular species composition of GlcCer suggested that inital Δ4-desaturation was followed by Δ8-desaturation and 9-methylation of the ceramide in fungi [78]. However, not clear is whether GlcCer formation occurs before or after the introduction of various functional groups into the ceramide backbone. The fact that free ceramides from several fungi contain 9-methyl-sphinga-4,8-dienine as a major component [169] supports the hypothesis that sphingoid base modifications precede GlcCer formation. However, whether free ceramides result exclusively from de novo synthesis or whether GlcCer degradation also occurs in fungi remains to be established. A glucosidase from *Phytophthora infestans* with sequence similarity to human glucosylceramidases (AF352032) did not show GlcCer-hydrolyzing activity in vitro [170]. Final glucosylation of completely processed ceramides is also consistent with the ER localization of all early steps of sphingolipid biosynthesis at least until Δ4-desaturation and the Golgi-localization of the GCS (in mammals). However, there is no unequivocal evidence for the proposed sequence of ceramide modification and finishing glucosylation.

A mouse melanoma cell line deficient in GCS activity shows that GlcCers are not essential for viability of isolated mammalian cells in culture [171]. However, mice with a disrupted GCS gene die during embryogenesis suggesting that GlcCers, or higher homologues, play a crucial role in cell differentiation and cell-cell interactions during embryogenesis [172]. In agreement with these studies, null mutants of single-cell organisms like the yeasts *P. pastoris* und *C. albicans* are viable and grow like the parental strains under different growth conditions [78]. Further aspects of GlcCer functions in fungi are discussed below.

Glucosylceramide galactosyltransferase

Lactosylceramide, βgal(1$\rightarrow$4)βglc-ceramide, is a well-known component of mammalian sphingolipids. cDNAs encoding UDP-galactose:glucosylceramide β-1,4-galactosyltransferase have been cloned from rat and human [173, 174]. In fungi, the occurrence of Gal_3Glc-ceramide and βgal(1$\rightarrow$4)βglc-ceramide has been proven for *N. crassa* [70] and *M. grisea* [77], respectively. To date, candidate sequences for glucosylceramide galactosyltransferase showing significant similarity to the mammalian enzymes have not been found by BLAST searches in finished and unfinished fungal databases.

Ceramide galactosyltransferase

Galactosylceramides have been found in animals and in some fungi, such as *Aspergillus fumigatus*, *Aspergillus nidulans*, *C. utilis*, *S. cerevisiae* and *S. schenckii* (table 1). The first cDNA encoding a UDP-galactose:ceramide galactosyltransferases was cloned from rat [175]. Deletion of the galactosyltransferase gene in mouse caused male infertility and severe dysmyelinosis in cells of the central and peripheral nervous system [176–178]. This galactosyltransferase also accepts diacylglycerol and alkylacylglycerol as substrates [178, 179]. Consequently, all galactolipids resulting from this activity, including the seminolipid 3-sulphogalactosyl-1-alkyl-2-acylglycerol, are not synthesized by the galactosyltransferase-deficient mice [178]. In contrast to the GCS, the ceramide galactosyltransferae is oriented toward the lumen of the ER [175, 180]. Only little information is available on the occurrence of galactosylceramides in fungi and none on their function or on the galactosyltransferase responsible for their formation. The structures of galactosylceramides

from *C. utilis* and *S. cerevisiae* have not been determined unambiguously. In contrast, structural analyses of sphingolipids from *A. fumigatus* and *S. schenckii* show that galactosylceramides and GlcCer possess an identical ceramide backbone, except for a higher proportion of Δ3-desaturation in the galactosylceramides [71, 75]. The mycelial form of *S. schenckii* contain only GlcCer whereas the yeast form contains both GlcCer and galactosylceramides in approximately equal amounts [75]. A gene responsible for the biosynthesis of galactosylceramides has not been cloned from any fungus. BLAST searches revealed an open reading frame on a genomic fragment (contig 961) of *A. fumigatus* showing low but significant similarity to the mammalian ceramide galactosyltransferases (preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org.

Functions of GlcCers in plants, fungi and animals

Before a detailed discussion of recently discovered functions of GlcCer in plants and fungi, we will present a limited selection of GlcCer functions in mammals which can be usefully compared with those of plants and fungi.

Mammals

Most GlcCer functions in mammals have been elucidated using (i) mutant cell lines impaired in GlcCer synthesis or degradation, (ii) genetically modified cells or mice deficient in GCS or overexpressing the corresponding gene and (iii) inhibitors of GlcCer synthesis. All these mutations or manipulations result in reduced or elevated GlcCer levels and, consequently, in quantitative alterations of its biosynthetic precursor ceramide and its oligoglycosylceramide downstream products. Thus, physiological effects are attributed either to GlcCer itself or to other sphingolipids.

The viability of a mouse melanoma cell line lacking glucosphingolipids shows that GlcCers are not essential for isolated mammalian cells in culture [171]. However, disruption of the GCS gene in mice resulted in lethal malfunctions during embryogenesis [172], suggesting that glycosphingolipids play a crucial role in cell differentiation and cell-cell interactions [181]. Down-regulation of GCS activity by antisense transfection reduced tumorigenicity of mouse melanoma cells [182] and increased the ceramide level without affecting apoptosis [183]. In a multidrug-resistant cancer cell line subjected to adriamycin stress, antisensing GCS led to apoptosis and restored adriamycin sensitivity [184]. The interpretation of this result is facilitated by the previous finding that some multidrug-resistant cancer cells contained increased amounts of GlcCer [185–188]. Formation of GlcCer seems to be one of several different mechanisms to escape apoptosis induced by increasing ceramide levels after treatment with cytotoxic drugs [5, 10]. Thus, reducing the ability of the cell to synthesize GlcCer by antisense transfection or by GCS inhibitors sensitizes them for some cytotoxic agents and reverses multidrug resistance, respectively [184, 189–192]. Consequently, in a reverse approach, overexpression of GCS resulted in enhanced multidrug resistance [189, 193]. Another study came to the contrary observation that overexpression of GCS did not affect apoptosis [8, 194]. The increased enzyme activity resulted in elevated GlcCer formation from de-novo-synthesized ceramide in the Golgi apparatus but had no access to ceramide released from sphingomyelin in the PM. There is no obvious explanation for these inconsistent data. There may be a regulatory connection between ceramide in the PM and the Golgi-located enzyme, since ceramide released from sphingomyelin by exogenous sphingomyelinase induced transcriptional up-regulation of GCS [195, 196]. GCS likely contributes to the regulation of excess ceramide levels at least in some cell types and compartments although the mechanism is still unknown. Accumulation of GlcCer can also be achieved by uncoupling GlcCer and lactosylceramide biosynthesis in the Golgi apparatus [188] and by impairment of GlcCer degradation through inhibition or mutagenesis of glucocerebrosidase [197, 198]. These latter studies also contribute to the understanding of Gaucher disease [199]. Regarding possible functions of GlcCer in the context of an individual cell growing in isolation and without contact with neighbours, experiments with a melanoma cell line are of utmost importance. Sprong and coworkers [200] showed that melanoma cells deficient in glucosphingolipid synthesis do not synthesize melanin pigment. This finding was explained by the impaired transport of a key enzyme in melanin biosynthesis, tyrosinase, from the Golgi apparatus to melanosomes and showed that glycosphingolipids were involved in this process [200]. If this effect is not only limited to tyrosinase and melanoma cells, this observation may be very significant (see below).

Plants

There is little information on the functions of sphingolipids and GlcCer in plants. Recent studies indicate that sphingolipids are involved in signal transduction not only in animals and fungi, but also in plants. The knockout of an *Arabidopsis* gene encoding a sphing-4-enine transfer protein caused activation of programmed cell death [201]. Furthermore, sphing-4-enine-1-phosphate was identified in plants in very low amounts, and its involvement in signal transduction linking the perception of the plant hormone abscisic acid to stomatal pore regulation was demonstrated [202]. Hydrogenation of the Δ4-

double bond resulted in sphinganine-1-phosphate, which was inactive. Remarkably, more prominent sphingolipids like GlcCer and GIPC contain only minute amounts of the sphingobase sphing-4-enine (see above). This shows that low abundance of particular sphingoid bases points either to their selective incorporation into rare sphingolipids or the exclusive occurrence in specific cell types and tissues, but does not indicate biological insignificance.

There is no direct evidence for any function of GlcCer in plants. For insight into membrane lipid functions in general, many studies have been performed investigating alterations in lipid composition as a consequence of environmental stress conditions as e.g. chilling or freezing stress, drought and toxic concentrations of metal ions (table 2). These studies have been greatly improved from rather simple determinations of only phospholipids and sterols from leaves towards comprehensive analyses of the molecular species of each lipid from purified vacuolar or plasma membranes. However, interpretation of their results remains difficult. In some cases, distinguishing between stress-induced alterations of lipid composition resulting from impaired lipid metabolism on the one hand and controlled adaptation mechanisms of the plant maintaining membrane functions under stress conditions on the other hand is difficult. Thus, alterations of lipid composition have to be assessed in terms of the contribution of each lipid to the physical and physiological properties of the membrane, resulting in positive or negative effects on membrane function under the respective stress condition. However, lipids determine many different properties of the membrane such as fluidity, permeability, bilayer stability (propensity to form a bilayer or inverted micelles), the temperature-induced liquid crystalline to gel phase transition, surface hydration, lateral lipid mixing or demixing and activity of intrinsic membrane proteins.

Despite the complexity of the problem, some of these studies provided valuable hints on the function of GlcCer in plant membranes, and freezing injury of plants is exemplified briefly below. Different studies have observed that the proportion of GlcCer in the PM of freezing-tolerant plants is lower compared to freezing-sensitive plants and that the GlcCer content decreases during the process of cold acclimation [51, 203, 204] (table 2). In addition, the molecular species composition of GlcCer differs between sensitive and tolerant plants and changes during cold acclimation towards increased proportions of monounsaturated VLCFAs, and more cis-Δ8 double bonds at the cost of trans double bonds in the sphingoid base moiety of GlcCer from tolerant plants [43, 44, 50, 51, 203].

Disruption of the PM is a primary cause of freezing injury [205, 206]. If extracellular ice formation occurs depending on temperature and solute concentration, lethal intracellular ice formation will be absent as long as the PM remains intact. The chemical potential of ice depends directly on the subzero temperature forcing the intracellular solution to come into equilibrium with the extracellular ice. This results in water flux though the PM, and dehydration of the plant cell continues until intracellular osmolality is equivalent to the chemical potential of extracellular ice. Thus, freezing stress manifests itself in cell dehydration, and survival of the cell depends mainly on the ability of the PM to withstand cell dehydration during extracellular ice formation and cell expansion during thawing. Physical studies on GlcCer revealed that this lipid shows properties which are deleterious to the PM under the described conditions [205, 207–209]. GlcCers are the least hydrated lipid species in the PM and enhance their propensity to undergo inverted-micelle formation which destroys the bilayer structure leading to lethal intracellular ice formation. Although membrane properties obviously cannot be attributed to one lipid component, these studies show that low GlcCer contents in the PM may contribute to their stability under freezing stress. The ability to genetically manipulate plant lipid composition will support further investigations into the role of lipids and GlcCer in freezing injury.

A new field, where GlcCer functions may soon be studied at the molecular level, comprises plant-pathogen interactions. Fungal GlcCer elicited hypersensitive cell death and phytoalexin accumulation in plants [210–212]. These GlcCer-induced plant defence responses protected rice against infection by the pathogenic fungus *M. grisea*, the causal agent of rice blast disease [211]. The methyl group at C-9 and the trans-Δ4 double bond in the sphingoid base moiety of fungal GlcCer were essential for elicitor activity. The glucose headgroup was not crucial, since free ceramides were also effective elicitors. GlcCers are likely also involved in other host-pathogen relationships, since the human pathogen *Helicobacter pylori* binds to gluco- and galactosylceramides in vitro [213].

Fungi

The first evidence for GlcCer functions in fungi was obtained by Kawai and coworkers who found that fungal GlcCers show fruiting-inducing activity in the fungus *Schizophyllum commune* [214]. Structural analysis of many different GlcCers and their derivatives with subsequent determination of their activity revealed that a cis- or trans-Δ8 double bond but not a Δ4 double bond in the sphingoid base moiety was essential. GlcCers containing fatty acid moieties of 14–18 carbons show stronger activity than C_{20} and C_{22} GlcCer while those with C_{24}-C_{26} were inactive. In this process, the methyl group at C-9 of the sphingoid base and the glucose headgroup were not cruical [80, 81, 214, 215]. The fruiting body induction by GlcCer was suggested to be due to their inhibitory effect on replicative DNA polymerases [216].

Although exploration of glycosphingolipid functions in fungi is in its infancy, recent studies have provided new insights into inositol-containing sphingolipids. *S. cerevisiae ipt1* mutant cells lacking mannosyl diinositolphosphoryl ceramide are resistant to two antifungal compounds, syringomycin from the bacterium *Pseudomonas syringae* [217] and a plant defensin from *Dahlia merckii* [218]. These findings suggest that mannosyl diinositolphosphoryl ceramide is the target of these antifungal agents, but a putative binding to the lipid at the PM of fungal cells remains to be demonstrated. Another gene, *IPC1*, encoding the inositol phosphoryl ceramide synthase, is required for melanin pigment biosynthesis in the human pathogen *Cryptococcus neoformans* [219] and for polarized cell growth of *A. nidulans* [220]. Of interest, obviously, would be to compare these data with similar investigations on GlcCer, to distinguish specific functions of the different polar headgroups and to identify the roles of VLCFAs which are the sole fatty acids in inositol-containing sphingolipids but are rarely present in GlcCer. A *P. pastoris* GCS-null mutant was not resistant to syringomycin indicating a specific interaction of the antifungal agent with mannosyl diinositolphosphoryl ceramide [J. Takemoto, personal communication]. Studies on the functions of GlcCer in melanin pigmentation have not been performed with fungi, but the above-mentioned mammalian albino cells deficient in GCS emphazise the importance of such investigations. Besides *C. neoformans*, phytopathogenic fungi like *M. grisea*, which require melanin for proper function of specialized infection structures called appressoria [221, 222] are also interesting organisms to be studied in this context.

Hints on GlcCer function in fungal development have been obtained using GCS inhibitors [223] and by the recent generation of different anti-GlcCer antibodies [224–227]. Both inhibition of GCS in *A. nidulans* and *A. fumigatus* [223] as well as incubation of *C. neoformans*, *Pseudoallescheria boydii* and *C. albicans* with anti-GlcCer antibodies inhibited morphological transitions such as budding and germ tube formation [225, 227]. Interestingly, the antibodies discriminate different morphological forms of the fungi. A monoclonal antibody allowed staining of the conidia from *A. fumigatus* and the yeast forms of *P. brasiliensis*, *H. capsulatum* and *S. schenkii*, but not of *C. albicans*. The mycelial forms of *P. brasiliensis* and *A. fumigatus* showed only weak staining [226].

In contrast, anti-GlcCer antibodies purified from rabbit serum stained filamentous forms of *P. boydii*, but not conidia [227]. Purified human antibodies accumulate mostly on budding sites of dividing *C. neoformans* cells and predominately localized to the cell wall [225]. This context requires mentioning that GlcCers have been isolated from both yeast and filamentous forms of several fungi in approximately equal amounts. Thus, differences in staining efficiencies may either be due to local accumulation of the glycolipids or to variable access of the antibodies to their targets due to alterations in cell wall organization [226].

Both immunolocalization of GlcCer and inhibition studies suggest that these glycolipids play a role in fungal cell differentiation. In contrast, a *C. albicans* GCS-null mutant was viable and still able to grow in filamentous form [78]. Thus, one can predict that this strain is able to compensate for hypothetical GlcCer functions in cell differentiation and should be resistant to both GCS inhibitors and anti-GlcCer antibodies. If it turns out to be still sensitive for such a treatment, the conclusion must be that the inhibitors and the antibodies recognize additional targets different from GlcCer.

In summary, the availability of most genes involved in GlcCer biosynthesis will enable the generation of various mutants of several fungal species and of the plant *A. thaliana*. These mutants will enable a new approach for studying GlcCer functions in these organisms. Single-cell yeasts will serve as model organisms to improve our knowledge about GlcCer functions in individual cells. First evidence that GlcCers are involved in intracellular transport processes have been obtained from mammalian cells [200]. Furthermore, studies with plants and fungi will include the roles that GlcCers play in cell-cell interactions. Such glycosphingolipid-dependent interactions between plants and fungi were discovered unexpectedly. On the one hand, plants recognize fungal GlcCer and activate antifungal defence mechanisms as a response [210]. On the other hand, one of several defence mechanisms, a plant defensin, is directed against fungal glycosphingolipids [218]. In contrast to animal cells which are able to interact with each other by direct contact of their PMs and their glycocalix, GlcCer-dependent plant-fungal interactions happen despite the fact that cell walls surround the cells of both organisms. Future studies will examine the mechanism of host-pathogen relationships and will show whether glycosphingolipids are also involved in the interaction between mammals and pathogenic fungi.

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