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Review

Plant sphingolipids: structural diversity, biosynthesis, first genes and functions

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Abstract

In mammals and *Saccharomyces cerevisiae*, sphingolipids have been a subject of intensive research triggered by the interest in their structural diversity and in mammalian pathophysiology as well as in the availability of yeast mutants and suppressor strains. More recently, sphingolipids have attracted additional interest, because they are emerging as an important class of messenger molecules linked to many different cellular functions. In plants, sphingolipids show structural features differing from those found in animals and fungi, and much less is known about their biosynthesis and function. This review focuses on the sphingolipid modifications found in plants and on recent advances in the functional characterization of genes gaining new insight into plant sphingolipid biosynthesis. Recent studies indicate that plant sphingolipids may be also involved in signal transduction, membrane stability, host–pathogen interactions and stress responses.
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1. Introduction

Sphingolipids are ubiquitous membrane components in eukaryotic cells and in a few bacteria [1,2]. Their chemical structure differs from the more commonly known glycerolipids in having a ceramide backbone, which consists of a fatty acid attached to a long-chain amino alcohol. Recent interest is focussing on the role of sphingolipids in serving as intra- and intercellular second messengers regulating cell growth, differentiation, apoptosis, and pathogenic defense [3,4]. Compared to the tremendous research on bioactive sphingolipids in mammalian systems and *Saccharomyces cerevisiae* published during the last two decades, there is a paucity of studies using plant systems. Studies on sphingolipid metabolism in plants have focused on demonstrating and characterizing the in vitro activities of enzymatic steps in major pathways [5]. The success in elucidating additional aspects of their metabolism and in recognizing first functions are mainly due to the fact that genes controlling crucial steps in the biosynthesis of sphingolipids have been identified only recently from plants and some other phyla.

These notable and recent advancements in the knowledge of plant sphingolipid biosynthesis and function will be summarized here together with an indication of remaining gaps and possible future research directions. In many studies, *S. cerevisiae* served as a model organism to study sphingolipid metabolism and seems to become the first eukaryotic organism in which all sphingolipid metabolic genes are identified. However, this statement does not apply to the biosynthesis and functions of the structural diverse plant sphingolipids. Indeed, divergencies in the biosynthetic pathway of plants lead to cerebrosides and glycosyl inositol phosphorylceramides (GIPC) with a preference for Δ8-unsaturated long-chain bases (LCB) [6–12] not present in baker's yeast. Despite or just because of the exceptional absence of cerebrosides and unsaturated LCB in yeast, this organism has been successfully used for the functional identification of heterologously expressed genes involved in plant sphingolipid synthesis. In this way, genes encoding enzymes modifying the ceramide core have been recently identified from a variety of different phyla including plants [13–17]. Sequence comparisons shed new light on evolutionary relationships of some of these proteins [16,18]. An

Abbreviations: ER, endoplasmic reticulum; GCS, glucosylceramide synthase; GIPC, glycosyl inositol phosphorylceramide; LCB, long-chain base; SAM, sphinganine-analogous mycotoxins

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extrapolation of the success with *S. cerevisiae* as a model suggests that the generation of plant mutants affecting sphingolipid metabolism will promote the elucidation of sphingolipid functions. Targeted gene disruption by homologous recombination established for the moss *Physcomitrella patens* [19,20], RNAi antisense inactivation [21,22] and the identification of transposon-tagged *Arabidopsis thaliana* mutants [23,24] will provide unprecedented opportunities to identify and characterize new functions for plant sphingolipids.

In the first part, this review will give an overview of the structures of sphingolipids found in plants, thereby placing emphasis on seemingly minor structural modifications compared to other phyla which may have unexpected relevance on sphingolipid functions. Based on the recent progress in understanding the molecular biology of sphingolipid biosynthesis, we will then develop a current picture of this pathway in plants. The knowledge of the functions of sphingolipids and their precursors in plants has just started to advance [25–27]. We will summarize recent data from this field trying to focus on features of plant sphingolipids not covered by previous reviews [5,25–29].

2. Structural diversity of plant sphingolipids

Sphingolipids are generated by the addition of a polar head group to ceramides which in turn are composed of a LCB (2-amino-1,3-dihydroxyalkane) carrying a N-acylated fatty acid of 14–26 carbon atoms. Complex sphingolipids, such as cerebrosides and GIPC (phytoglycolipids) are formed by the addition of various glycosyl residues and other polar phosphate-containing headgroups to the ceramide. Depending on the source, this basic ceramide structure can be modified by differences in chain length, methyl branching, insertion of additional hydroxy groups, and degree of unsaturation [1].

2.1. Long-chain bases

In mammals, the LCB moiety is mostly (*E*)-sphing-4-enine (sphingosine, d18:1^{4}), whereas in the yeast *S. cerevisiae*, the predominant LCB is 4-hydroxysphinganine (phytosphinganine, t18:0) formed by the desaturation or hydroxylation of sphinganine (d18:0) at C-4, respectively. In contrast, the sphingoid base composition of plants is more variable, being composed of up to eight different C_{18}-sphingoid bases derived from D-erythro-sphinganine (Fig. 1). Due to an additional *cis*- or *trans*-desaturation at C-8, the predominating regioisomers of unsaturated plant LCB are (*E*/*Z*)-sphing-8-enine (d18:1^{8}), (4*E*,8*E*/*Z*)-sphinga-4,8-dienine (d18:$2^{4,8}$) and (8*E*/*Z*)-4-hydroxy-8-sphingenine (t18:1^{8}), whereas d18:1^{4} is virtually absent and d18:0 and t18:0 are only present in minor proportions [6–12]. Other LCB differing in chain length are present as minor components in plant sphingolipids [1]. In *Euphorbia characias* as well as in several other organisms including fungi, saturated and Δ6-(*Z*)-unsaturated tetrahydroxysphingenine derivatives may occur [30,31], suggesting the presence of an additional C5-LCB hydroxylase. The occurrence of a Δ6-(*Z*)-double bond may be either due to the activity of an "exotic" LCB-desaturase or to a serine palmitoyltransferase accepting Δ4-(*Z*)-myristoyl-CoA, if present in these organisms. The occurrence of Δ8-unsaturated LCB is not restricted to plants. In contrast to *S. cerevisiae*, which in this context has to be considered as a rare exception, many fungi mainly contain a di-unsaturated, methyl-branched LCB, (4*E*,8*E*)-9-methyl-sphinga-4,8-dienine in their cerebrosides [32–35], whereas t18:0 is the predominating LCB in GIPC [36–38]. In marine invertebrates, such as ascidians [39], echinoderms [40,41], sponges [42], bryozoa [43], sea anemones [44] and gorgonians [45], glycosylceramides and related compounds containing a tri-unsaturated, methyl-branched (4*E*,8*E*,10*E*)-9-methyl-sphinga-4,8,10-trienine have been detected. In gliding myxobacteria, ceramides with a (4*E*,8*E*)-17-methyl-sphinga-4,8-dienine were identified [46]. Apart from plants, a Δ8-*cis*-isomer has been only detected in the galactosylceramides of annelids, showing that leech has a non-branched, tri-unsaturated (4*E*,8*Z*,11*Z*)-docosasphinga-4,8,11-trienine [47]. As known so far, the occurrence of both Δ8 *cis*/*trans*-isomers seems to be restricted to plant sphingolipids. Ratios of Δ8-*trans*- to Δ8-*cis*-isomers varying from 91:9 in cucumber to 4:86 in wheat have been found in the leaf cerebrosides of different plant species [6]. Interestingly, in *A. thaliana*, the (8*Z*)-t18:1 is the most abundant LCB in leaf cerebrosides recovered from lipid extracts [8], whereas direct alkaline hydrolysis of whole leaves indicates that the (8*E*)-t18:1 isomer is most abundant [13]. Taking into account that GIPC are hardly extractable in organic solvents such as chloroform/methanol [48], which are suitable for the extraction of most membrane lipids including cerebrosides, these data suggest that other complex sphingolipids such as GIPC must be more abundant than monoglucosylceramides in *A. thaliana* leaves. The analysis of these complex phytoglycolipids is handicapped by complicated extraction and purification procedures and only a few plant GIPC have been analysed in detail [49–54]. Therefore, at present, no exact data on the quantitative proportions of cerebrosides and GIPC in plants are available. A comparison of the LCB compositions of *A. thaliana* leaves derived from lipid extracts (cerebroside fraction) and from lipid-depleted tissues (GIPC fraction) are pointing to a predominance of GIPC compared to cerebrosides and confirms the suggestion mentioned above that there is a channelling of the (8*Z*)-t18:1 into cerebrosides and of the (8*E*)-isomer into GIPC (P. Sperling and E. Heinz, unpublished data). Furthermore, the relative proportions of di- and trihydroxybases in cerebrosides differ with plant species as well, that is, from 78% dihydroxybases in soybean [55] to 87% trihydroxybases in *A. thaliana*, whereas the glucosylceramides of leaf and root tissues have similar LCB compositions [8]. These studies suggest that plants maintain two separate ceramide pools for the biosynthesis of cerebrosides

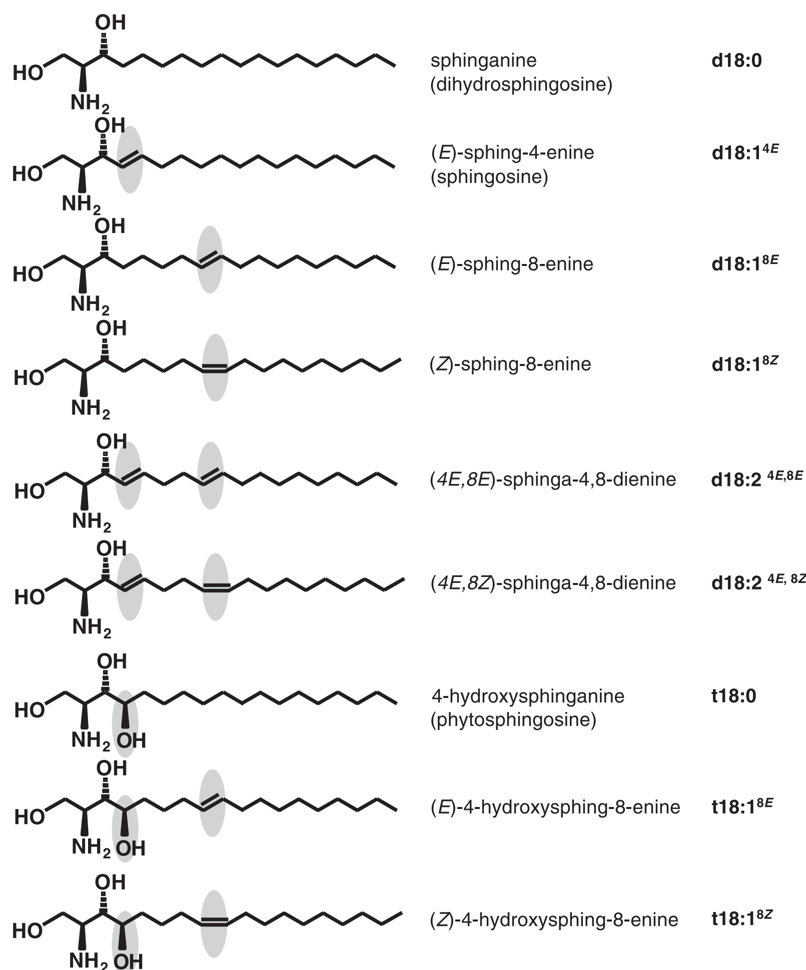


Fig. 1. Structures of common long-chain bases from plants. Names and shorthand designations according to Karlsson [1] are given for each sphingoid base. For certain bases, the trivial name is also given in parentheses. All naturally occurring dihydroxy sphingoid bases have D-*erythro* and all trihydroxy sphingoid bases have D-*ribo* configuration. Additional double bonds and hydroxy groups characterizing the sphingoid bases derived from sphinganine are marked by grey background. Only common C_{18}-sphingoid bases are depicted in the figure, although long-chain bases of different chain lengths occur in minor amounts. In plant cerebrosides, the 8-(*E*)- and 8-(*Z*)-isomers of sphinga-4,8-dienine and 4-hydroxy-sphinga-8-enines represent the dominant bases [6–12,103], whereas sphingosine and 4-hydroxysphinganine, which are the predominant long-chain bases in animals and *S. cerevisiae*, respectively, have been detected only in minor amounts. GIPC and GPI anchors with 4-hydroxysphinganine and 4-hydroxysphing-8-enine have been isolated from plants [49–52,77], but their LCB compositions have not yet been assigned completely.

and GIPC, which could be achieved by different ceramide selectivities of glucosylceramide synthase (GCS) and inositol phosphorylceramide synthase or by restricting access of the enzymes to spatially separated substrate pools.

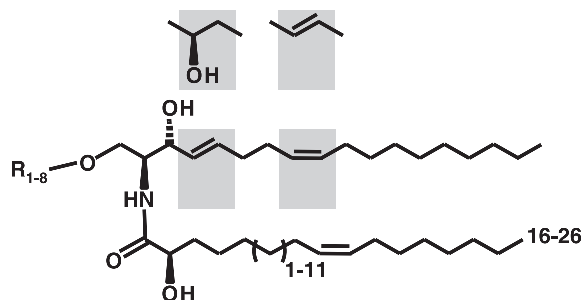
2.2. Fatty acyl amides

In the ceramide backbones of plant sphingolipids, more than 10 different fatty acids can be N-acylated to the eight different LCB mentioned above. These fatty acids are almost exclusively α-D-hydroxylated and vary in their chain lengths from C_{14} to C_{26} including significant, but not predominating chains of odd carbon numbers [28,56–58]. Saturated C_{16}, C_{20}, C_{22} and C_{24} α-hydroxylated fatty acids are most abundant, whereas ω9-monounsaturated very long-chain fatty acids ranging from C_{22} to C_{26} occur in low proportions [57]. The occurrence of 2-hydroxy nervonic acid (24h:1) is characteristic for the leaf cerebrosides of some chilling-resistant cereals [28,59]. The occurrence of ω9-*cis*-unsaturated very long-chain fatty acyl amide residues in sphingolipids may be attributed to the sequential fatty acid elongation of oleoyl-CoA resulting in a series of ω9-monounsaturated very long-chain fatty acyl-CoA of 22–26 carbons which could serve as substrate for the plant ceramide synthase. In free ceramides, non-hydroxylated fatty acids can account for 1–32%, whereas in leaf cerebrosides, they are minor constituents ranging from 1% to 3% [11,60,61]. In several plants, even 2–3% of 2,3-dihydroxy fatty acids have been detected [62,63], suggesting the existence of a regio-unselective acyl amide α-hydroxylase or of a C3-hydroxylase. In yeast GIPC, fatty acids of 26 carbons in length are predominating, the majority of which is hydroxylated at the α-position [64,65]. The presence of 2,3-dihydroxy acids has been described in *N*-acyl-4-hydroxysphinganine isolated from *S. cerevisiae*

[66,67], whereas a Δ3-(*E*)-unsaturation of 2-hydroxy fatty acids appears to be a modification restricted to some fungal cerebrosides [32,68,69]. These data indicate not only the presence of an acyl amide C3-hydroxylase in plant and baker's yeast, but also of an acyl amide Δ3-(*E*)-desaturase in some pathogenic fungi. The corresponding genes coding for these modifications at C-3 have not been identified yet. In mammalian cerebrosides, typically (*E*)-sphing-4-enine is linked to fatty acids of 16–24 carbons, which may be α-hydroxylated. However, in some tissues such as human epidermis, the ceramides contain 4-hydroxysphinganine and unusual fatty acids with up to 34 carbons, which occur in non-hydroxylated and ω-hydroxylated forms [70,71].

2.3. Polar head group

Studies on the molecular species of ceramide residues show that almost all possible combinations of LCB and fatty acids occur in nature giving rise to two types of complex plant sphingolipids (Fig. 2). In plants, neutral cerebrosides carry one to four glycosyl residues attached to the primary hydroxyl group of the sphinganine derivatives, whereas in the negatively charged GIPC (phytoglycolipids), inositol-1-phosphate is linked as a phosphodiester to C-1 of the ceramide backbone, which may be further extended by oligosaccharide chains. Apart from a few studies of GIPC from tobacco [51–53] and corn [49], neither their complex core structure nor their biosynthesis has been studied in detail in plants [54]. GIPC have been shown to occur as membrane anchors (GPI anchors) of covalently bound proteins in yeast [72], mammals, protozoa [73] and plants [74–79].



Ceramides
R_1= H-

Cerebrosides
R_2 = β-D-Man-
R_3 = β-D-Glu-
R_4 = [β-D-Man(1,4)]$_{1-3}$-β-D-Glu-
R_5 = β-D-Glu(1,4)-[β-D-Man(1,4)]$_{1-2}$-β-D-Glu-

GIPC (phytoglycolipids)
R_6 = α-D-GluN(1,4)-α-D-GluA(1,2)-*m*-Ino(1-P)-
R_7 = β-D-Gal(1,4)-α-D-GluNac(1,4)-α-D-GluA(1,2)-*m*-Ino(1-P)-
R_8 = [α-D-Man(1,2)]-α-D-GluN(1,4)-α-D-GluA(1,6)-*m*-Ino(1-P)-

Fig. 2. Structures of ceramide glycosides from plants. The ceramide backbones of plant sphingolipids show a large variability. Their long-chain bases carry four possible modifications (on grey background), that is, hydroxylation or (*E*)-desaturation at C-4, and (*E*)- or (*Z*)-desaturation at C-8 resulting in seven frequent LCB, also shown in Fig. 1. These LCB are linked to more than 10 different fatty acyl groups, which in turn vary in α-hydroxylation, chain length and ω9-unsaturation. Cerebrosides are formed by glycosylation of the C1-hydroxy group of ceramide (R_1) yielding β-D-glucosyl and β-D-mannosyl ceramide (R_{2-3}). The glucosyl derivative may by elongated by sequential addition of up to three β-1,4-mannosyl residues resulting in oligosaccharides terminally capped by a β-1,4-glucosyl residue (R_{4-5}). Glycosyl inositol phosphorylceramides (GIPC, phytoglycolipids) isolated from tobacco leaves (R_{6-7}) and corn kernels (R_8) carry a 1-phospho-*myo*-inositol residue linked via C2 and/or C6 to C1 of an α-glucuronosyl residue [49,52,53]. Additional glycosyl derivatives with further galactosyl, mannosyl and fucosyl residues have been isolated from R_{7-8}.

The structural features of cerebrosides in plants have been analysed in detail (reviewed in Ref. [29]). There are two different monoglycosyl ceramides in plants carrying either β-D-mannosyl- or β-D-glucosyl residues. The glucosylceramide is mainly used for further β(1→4) linked mannosylations resulting in series of di-, tri- and tetraglycosyl ceramides, which are terminally capped by a glucosyl residue apparently preventing further chain elongation [80,81]. Therefore, it seems unlikely that the cellobiosyl ceramide may act as a primer for cellulose synthesis as proposed for β-sitosterol glucosides in plants [82]. More than 20 different glucosylceramide species with 12 species comprising each more than 1 mol% of the total cerebroside mixture have been determined in some plant species [60,83–87]. Galactosyl ceramides, neuraminic (sialic) acid-containing ceramides (gangliosides) and sphingomyelin, all of which are typical mammalian sphingolipids [88], have not been found in higher plants. Most fungi contain two types of sphingolipids, cerebrosides, with mainly glucosyl or galactosyl residues, and GIPC with mannosyl residues [89], while baker's yeast represents one of the few exceptions containing GIPC but no cerebrosides. In plants, the glucosylceramides typically account for less than 5 mol% of the total lipids, but are quantitatively important components of the outer (apoplastic) monolayer of the plasma membrane comprising 7–30 mol% of membrane lipids [90]. On the other hand, the bilayer distribution of sphingolipids in the tonoplast has not been determined yet. The same is true for the intracellular location and transbilayer distribution of GIPC in plants, which remain to be investigated and quantified, as outlined above. The sum of both, cerebrosides and GIPC, may be significantly higher than anticipated. If they are concentrated in one leaflet of bilayer membranes, the proportion of phospholipids would be significantly reduced.

3. Characterization of plant genes for sphingolipid biosynthesis

In the following, recent progress in the cloning and identification of genes involved in sphingolipid biosynthesis of plants will be discussed with emphasis on the synthesis of the characteristic unsaturated LCB. A scheme for the

proposed pathway of sphingolipid synthesis in plants is shown in Fig. 3. Many of the proteins involved still need further biochemical characterization, in particular their subcellular localization in the plant cell. For an easier comparison, the gene names from *S. cerevisiae* are included in Fig. 3, most of which have already been functionally characterized. The identified orthologous plant genes (in most cases missing their own nomenclature) are indicated by black boxed enzyme names. To encourage future characterizations of presently putative plant genes, sequences identified in the genome of *A. thaliana* are included in the following compilation [ArabidopsisGenomeIntitiative 2000] and are marked by black dots in Fig. 3.

3.1. Ceramide synthesis

Sphingolipid biosynthesis starts with the condensation of acyl-CoA (mainly palmitoyl-CoA) and L-serine to yield 3-ketosphinganine, catalyzed by the palmitoyl-CoA:L-serine *C*-palmitoyltransferase (EC 2.3.1.50). Detection of this enzyme activity in plant microsomes by in vitro assays points to a localization in the endoplasmic reticulum (ER) [5]. The reaction can be specifically inhibited by L-cycloserine, β-chloro-L-alanine and by the antifungal agents sphingofungin B and C [5,91]. As shown for the yeast enzyme [92], the serine palmitoyltransferase from plants may also consist of two essential subunits, Lcb1 and Lcb2. An *LCB2* cDNA from *A. thaliana* has been functionally expressed in a yeast mutant defective in serine palmitoyltransferase activity [93]. Expression of a green-fluorescent protein fusion product in tobacco cells showed that Lcb2 is localized in the ER. Inspection of the complete *Arabidopsis* genome database suggests a second hypothetical *LCB2*-like gene (AB074928) [93] and a putative *LCB1*-like gene (AB063254). The serine palmitoyltransferase has a strong preference for palmitoyl-CoA, but also palmitelaidoyl-CoA with a *trans*-double bond at C-9, was still an effective substrate. Saturated acyl-CoAs of shorter or longer chain length as well as palmitoleoyl-CoA with a *cis*-double bond at C-9 were highly discriminated [94]. The specificity of the plant serine palmitoyltransferase for unsaturated C18 acyl-CoA such as oleic-, linoleic- or linolenic-CoA representing the main fatty acyl residues in plants, has not been investigated. It has been shown, that very long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (20:5) produced by the fungus *Mortierella alpina* are not incorporated into its cerebrosides [95].

In a second step, 3-ketosphinganine is reduced with NADPH by the D-*erythro*-sphinganine:$NADP^+$ 3-oxidoreductase (EC 1.1.1.102) to yield sphinganine (D-*erythro*-2-amino-1,3-dihydroxyalkane). The *TSC10* gene encoding this membrane-bound and essential enzyme in *S. cerevisiae* [96] has been located at the cytosolic side of the ER in mammalian cells [97]. Two homologs encoding putative 3-ketosphinganine reductases (NM_111481, NM_121925) can be found in the *A. thaliana* genome, but their functions have not been identified yet. In the next step, the amino group of sphinganine is acylated to yield ceramide (*N*-acyl sphinganine). In yeast, this reaction is catalysed by two similar acyl-CoA:sphinganine *N*-acyltransferases (ceramide synthases, EC 2.3.1.24), Lac1 and Lag1, requiring long-chain acyl-CoA [98,99]. Deletion of both genes is not lethal for *S. cerevisiae* but the cells grow very poorly. The ability of the double mutant to grow probably results from the ability of two ceramidases (EC 3.5.1.23), Ypc1 and YdcI, to also perform the reverse reaction and make ceramide using free fatty acids as substrate and representing an alternative, acyl-CoA-independent way for ceramide synthesis [98,99]. Two cDNA homologs of *LAG1* (AF198179, AF198180) may code for two putative sphinganine *N*-acyltransferases in *A. thaliana* [100]. The activity of such an enzyme has been demonstrated in microsomal membranes of squash, bean and corn, suggesting a localization in ER membranes [28]. D-*Erythro*-sphinganine and -sphingosine serve as substrates for the *N*-acyltransferase, whereas DL-*threo*-sphinganine and 4-hydroxysphinganine do not. In yeast, the ceramide synthases obviously channel C_{26} fatty acids into GIPC. In plants, the distribution of hydroxy fatty acyl chains in glucosylceramides is paralleled by the substrate specificity of the enzymes using C_{16} to C_{24} acyl-CoA [28]. These data suggest a key role for this enzyme in determining the acyl amide compositions of both cerebrosides and GIPC. Hydroxy acyl chains do not function as substrate, indicating that α-hydroxylation apparently occurs following ceramide formation [5].

The second mechanism for ceramide synthesis utilizing predominantly free palmitic acid in plants has been demonstrated in vitro [28]. Ceramidase activity is stimulated by the addition of *erythro*-sphinganine, whereas (*E*)-sphing-4-enine is a poor substrate and 4-hydroxysphinganine inhibits ceramide formation. In yeast, two ceramidases preferring either *N*-acyl sphinganine, Ydc1, or *N*-acyl 4-hydroxysphinganine, Ypc1, have been cloned [101,102]. From these two enzymes, Ypc1 is probably involved in sphingolipid degradation, showing acyl-CoA-independent reverse activity in ceramide formation [101,102]. A putative ceramidase is also present in *A. thaliana*, but the *YPC1* gene (BAB60897) has not been cloned. Comparison of the in vitro activities of the sphinganine *N*-acyltransferase and the reverse ceramidase in plant membrane preparations indicates that ceramide formation in vivo may occur predominantly by the acyl-CoA-dependent reaction [5]. The role of the reverse ceramidase in vivo remains unclear, though it may act as salvage mechanism for otherwise cytotoxic free fatty acids and LCB.

3.2. Modifications of the hydrophobic ceramide core

Once the ceramide backbone is established, the LCB and acyl amide residues are further modified by desaturations and/or hydroxylations to form the molecular species commonly found in plants. Only a few enzymatic activities required for these modifications have been demonstrated in

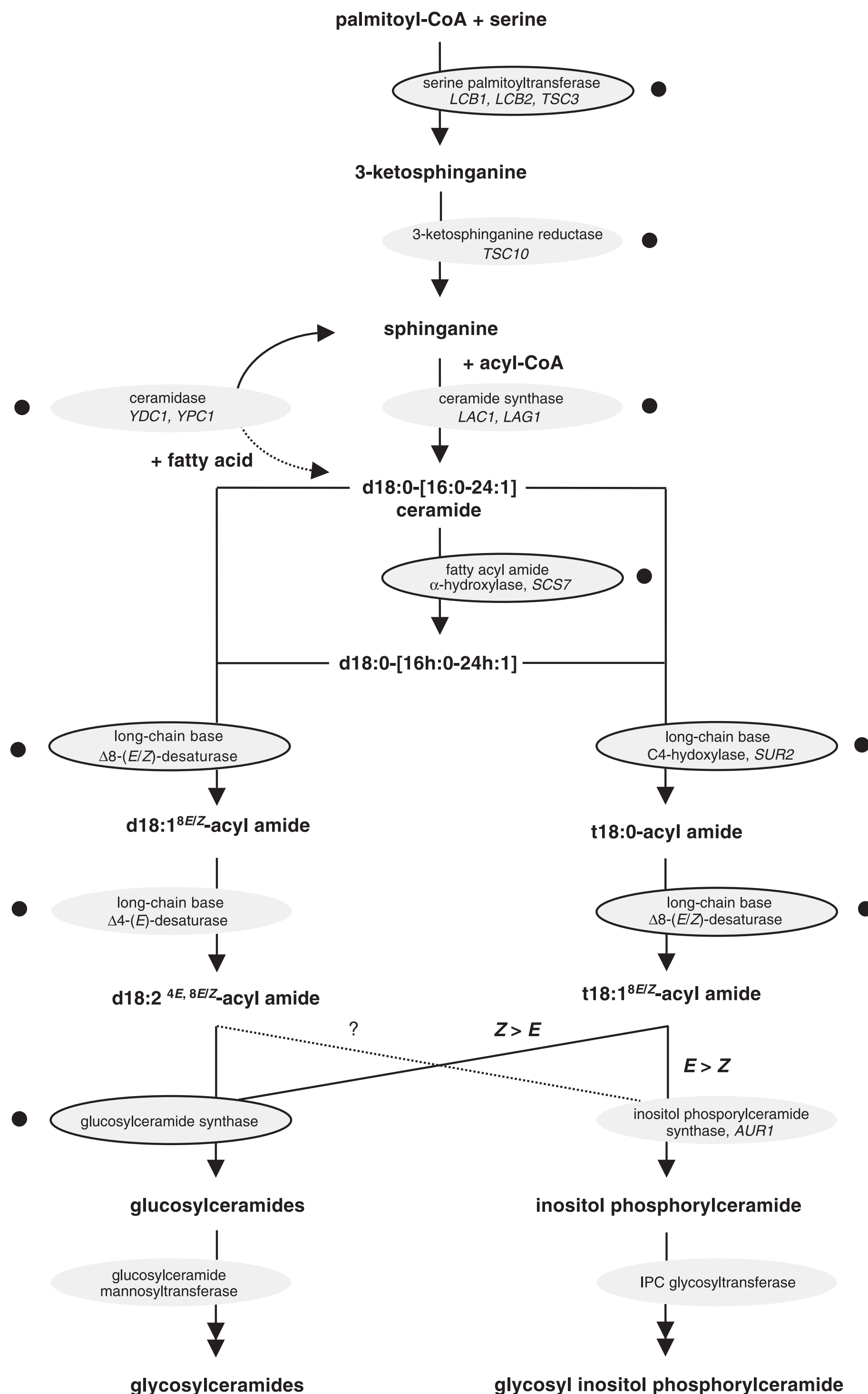


Fig. 3. Tentative pathway for sphingolipid biosynthesis in plants. Metabolites are shown in bold lettering, enzymes and their genes are included on grey background. Genes occurring in *S. cerevisiae* are given in the preferred designations listed in the Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces). Functionally identified plant genes are marked by black framing of the enzymes. The presence of putative *A. thaliana* genes identified by comparison of their deduced amino acid sequences is indicated by a black dot next to the enzyme. The placement of some steps of sphingolipid synthesis, in particular of Δ4- and Δ8-LCB desaturation, the substrate specificities of the involved enzymes (i.e. free ceramide, cerebroside and GIPC) and the channelling of molecular ceramide species into complex sphingolipids are hypothetical (see text for details). Reactions releasing metabolites involved in signalling such as sphingoid base phosphates, ceramide and lyso-sphingolipids are not depicted in this figure. Very long-chain fatty acids introduced into ceramides may be ω9-unsaturated [57].

vitro, and the true substrates for these reactions, that is, free LCB, ceramide, cerebroside or GIPC, are still not known with certainty. Where these modifications may operate in the biosynthetic pathway of plants is indicated in Fig. 3.

3.2.1. C4-LCB hydroxylation

In plants, sphinganine can be either desaturated to (*E*)-sphing-4-enine or it can be C4-hydroxylated to yield 4-hydroxysphinganine (phytosphinganine), most of which is further desaturated to yield *cis*/*trans*-isomers of Δ8-unsaturated LCB [6,103]. In *S. cerevisiae*, lacking Δ4-LCB desaturation, a non-essential *SUR2*/*SYR2* gene responsible for C4-LCB hydroxylation to give C_{18}- and C_{20}-phytosphinganine has been identified by gene disruption [104,105]. In yeast subjected to heat shock, a rapid but transient accumulation of free phytosphinganine preceding an increase in the level of ceramide has been observed, suggesting that free sphinganine can be C4-hydroxylated before incorporation into *N*-acyl sphinganine [106,107]. It is unclear whether sphinganine, *N*-acyl-sphinganine (dihydroceramide) or both are substrates for hydroxylation at C-4. *SUR2*-orthologous sequences have been found in *Schizosaccharomyces pombe*, *Candida albicans* and *A. thaliana* [14]. Heterologous expression of each of two Sur2-like genes identified from *A. thaliana* in a *sur2*Δ-null mutant lacking C4-LCB-hydroxylation resulted in the formation of D-*ribo*-C_{18}- and -C_{20}-phytosphinganine indicating the presence of two isoenzymes for C4-LCB hydroxylation in *A. thaliana* [14].

3.2.2. Δ4-LCB desaturation

In contrast to *S. cerevisiae*, plants [103], fungi [108] and mammals [109] synthesize sphingolipids with Δ4-*trans*-unsaturated LCB. In mammals, the Δ4-(*E*)-desaturation occurs at the cytosolic face of ER membranes at the level of *N*-acyl sphinganine [110–115]. NADH or NADPH and molecular oxygen are required as co-factors, whereas cyanide, divalent copper, dithiothreitol and antibodies raised against cytochrome b_5 inhibit sphingolipid Δ4-(*E*)-desaturase (dihydroceramide desaturase) activity [116]. Factors that influence the mammalian enzyme activity include the alkyl chain length of the LCB ($C_{18}>C_{12}>C_8$), the acyl amide chain ($C_8>C_{18}$), the stereochemistry of the LCB (D-*erythro* > L-*threo*-*N*-acyl sphinganines) and the nature of the headgroup with highest activity observed with *N*-acyl sphinganine, some with dihydrosphingomyelin, but no activity with free C_{18}-sphinganine or dihydroglucosylceramide [116].

The intermolecular primary isotope effects involved in dihydroceramide Δ4-desaturation have been determined by incubating rat liver microsomes with 4,4- and 5,5-dideuterated *N*-octanoyl-D-*erythro*-sphinganine, respectively, suggesting that initial oxidation of the substrate occurs at C-4 (see below) [117]. In a bioinformatics approach, sphingolipid Δ4-(*E*)-desaturase genes have been recently identified from *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster* and *C. albicans* followed by heterologous expression in a *S. cerevisiae sur2*Δ-mutant [16]. Man and mouse both possess a second Δ4-(*E*)-desaturase gene, from which the mouse gene turned out to code for a bifunctional sphingolipid Δ4-desaturase/C4-hydroxylase. Though not tested yet, the human homolog is probably also responsible for the synthesis of 4-hydroxysphinganine-containing sphingolipids because no Sur2-like sequence seems to be present in man. Plant orthologous Δ4-(*E*)-desaturase sequences have been found in *A. thaliana* (AF220201) and *Lycopersicon esculentum*, but their functional expression in *S. cerevisiae* has failed (J. Napier and P. Ternes, personal communications). In consideration of yeast lacking plant-specific molecular species of (glycosyl)ceramides, one possibility may be that this host does not provide the correct substrate for plant Δ4-desaturases. Both, the sphingolipid Δ4-(*E*)-desaturase as well as the *SUR2*-like C4-hydroxylase sequences show the three conserved histidine motifs characterizing all membrane-bound fatty acyl desaturases [118]. But both sequences do not contain a cytochrome b_5 domain, which inter alia has been found in sphingolipid Δ8-desaturases and some acyl amide α-hydroxylases (see below) [17,119].

3.2.3. Δ8-LCB desaturation

Like Δ4-(*E*)-desaturation, Δ8-unsaturated sphingolipids do not occur in baker's yeast. Interestingly, only the *trans*-isomers of Δ8-unsaturated LCB have been found in *Pichia pastoris*, *Rhynchosporium secalis*, *M. alpina* and other fungi [33,95], whereas a mixture of *cis*- and *trans*-isomers is characteristic for plants. Genes encoding plant sphingolipid Δ8-desaturases (EC 1.14.99) have been functionally identified from *A. thaliana*, *Brassica napus*, *Helianthus annuus* and *Borago officinalis* [13,120,121]. Surprisingly, heterologous expression of these cDNA sequences in *S. cerevisiae* resulted in significant proportions of both *cis*- and *trans*-isomers of plant characteristic 4-hydroxysphing-8-enines not present in wild-type yeast cells. The presence of C_{18}- and C_{20}-(8*E*/*Z*)-4-hydroxysphing-8-enines in these transgenic cells can be ascribed to the activity of a stereo-unselective sphingolipid Δ8-desaturase lacking absolute chain length specificity. Depending on the plant source, different and characteristic *E*/*Z*-ratios ranging from 3:1 to 7:1 were obtained when using the same yeast expression system. Any influence from an unspecific yeast isomerase could be excluded because wild-type yeast incorporating exogenously applied, synthetic Δ6-(*E*)-hexadecenoic acid into sphingolipids yielded exclusively (*E*)-4-hydroxysphing-8-enine and did not show any conversion to the (*Z*)-isomer (C. Beckmann and P. Sperling, unpublished data).

The formation of both (*E*)- and (*Z*)-double bonds results from a *syn*-elimination of two vicinal hydrogen atoms from two different substrate conformers [122]. Low but distinct kinetic isotopic effects suggest a preferential attack at C-8 of 4-hydroxysphinganine with *anti*-orientation en route to the *E*-isomer and at C-9 with *gauche*-orientation to the *Z*-isomer. Since both isomers are generated by the same

enzyme, a uniform mechanism involving a transient C-centered radical can be proposed. Therefore, the sphingolipid Δ8-desaturase is different from the hitherto studied and stereospecifically operating fatty acyl (*Z*)-desaturases including the sphingolipid Δ4-(*E*)-desaturase from rat, which all attack a hydrogen at the carbon atom proximal to the polar head [117,123].

The plant sphingolipid Δ8-desaturases tested in a yeast *sur2*Δ-mutant strain cultured with or without 4-hydroxysphinganine required a C4-hydroxylated substrate, suggesting that Δ8-desaturation followed C4-hydroxylation to yield (8*E*/*Z*)-4-hydroxysphing-8-enines [120]. However, non-hydroxylated (*Z*)- and (*E*)-sphing-8-enines are present in plant glucosylceramides [6,103] which may point to a second sphingolipid Δ8-desaturase activity in plants required for the synthesis of (4*E*,8*E*/*Z*)-sphing-4,8-dienines. In fact, a second sphingolipid Δ8-desaturase sequence is present in *B. officinalis* [121] and in *A. thaliana* (NM_130183). Expression of this second *A. thaliana* cDNA in a *S. cerevisiae sur2*Δ-mutant strain revealed a similar C4-hydroxy-preference of the enzyme, although traces of sphing-8-enine were also formed (P. Sperling and E. Heinz, unpublished data). These data are consistent with the high proportions of Δ8-unsaturated trihydroxybases but minor proportions of Δ8-unsaturated dihydroxybases present in *A. thaliana* sphingolipids [8,13].

Recently, expression of a sphingolipid Δ8-desaturase from *Aquilegia vulgaris* in *S. cerevisiae* and in a *sur2*Δ-mutant showed that this enzyme is able to use both 4-hydrosphinganine and sphinganine as substrates, respectively [124]. The LCB composition of this member of the Ranunculaceae has not been analysed yet. Therefore, it may be speculated that plant species containing higher proportions of (*E*/*Z*)-sphing-8-enine and (4*E*,8*E*/*Z*)-sphing-4,8-dienine express Δ8-desaturase isoenzymes differing in their selectivity for 4-hydroxysphinganine and sphinganine. Interestingly, expression of a moss cDNA in *S. cerevisiae* resulted in the first identification of a *cis*-specific sphingolipid Δ8-desaturase using 4-hydroxysphinganine as substrate (P. Sperling and E. Heinz, unpublished data). Furthermore, the activity of this Δ8-(*Z*)-desaturase is significantly increased in the presence of glucosylceramide. However, in plants, the sequence of hydroxylation and desaturation to form 4-hydroxysphing-8-enines and sphinga-4,8-dienes remains to be elucidated. Based on these data, a working hypothesis on the biosynthesis of unsaturated LCB in plants, assuming ceramide to be the main substrate, is shown in Fig. 3. In fungi such as *C. albicans*, *P. pastoris, Kluyveromyces lactis* and *Saccharomyces kluyveri*, putative sphingolipid Δ8-desaturases have been identified. Expression of the gene from *K. lactis* in *S. cerevisiae* leads to the conversion of 4-hydroxysphinganine to (*E*)-4-hydroxysphing-8-enine, whereas the gene from *S. kluyveri* was not active [125]. On the other hand, expression of a gene from *P. pastoris* in the yeast *sur2*Δ-mutant leads to the production of (*E*)-sphing-8-enine (P. Sperling and E. Heinz, unpublished data). In this case, the functional identification of a *trans*-specific sphingolipid Δ8-desaturase is consistent with the occurrence of (4*E*,8*E*)-9-methyl-sphinga-4,8-dienine in fungal cerebrosides lacking any *cis*-Δ8-double bonds. However, the specificities/selectivities of the plant and fungal Δ8-desaturases for free LCBs, ceramides, cerebrosides, GIPC or specific molecular species of these substrates, respectively, remain to be elucidated.

The sphingolipid Δ8-desaturase sequences identified in plants and fungi all show the histidine box motifs characteristic for membrane-bound desaturases [118] and their desaturase domain is *N*-terminally fused to cytochrome b_5 [119]. The identification of this *N*-terminal domain from sunflower has been confirmed by expression of the recombinant protein domain in *Escherichia coli* exhibiting redox absorbance spectra characteristic for plant microsomal cytochrome b_5 [126].

3.2.4. Acyl amide hydroxylation

Like fungi, sphingolipids from plants usually contain α-hydroxylated fatty acids. Evidence for a direct α-hydroxylation of fatty acyl residues only when they are bound as elements of intact sphingolipids or free ceramide came from radiolabeling studies of *Tetrahymena pyriformis* [127]. A non-essential acyl amide α-hydroxylase gene, *FAH1* or *SCS7*, respectively, has been identified in *S. cerevisiae* by gene disruption/deletion leading to a significant reduction in 2-hydroxylated cerotic acid (26h:0) [17,104]. Database searches revealed orthologous sequences from *S. pombe*, *Caenorhabditis elegans* and *A. thaliana*. Heterologous expression of one (AY050326) of the two *A. thaliana* homologs found in the genome, led to a significant increase in 26h:0 in a *fah1*Δ yeast mutant strain [17]. Whether the second homolog (AY058151) codes for an isoenzyme of the acyl amide 2-hydroxylase or for a 3-hydroxylase, responsible for the formation of 2,3-hydroxylated acyl amides as mentioned above, remains to be determined. Interestingly, the yeast and *C. elegans* protein sequences each showed a *N*-terminal cytochrome b_5-fusion which is lacking in the *A. thaliana* and *S. pombe* orthologs.

3.2.5. Phylogenetic relationships

The four different groups of enzymes [acyl amide α-hydroxylase, LCB C4-hydroxylase, LCB Δ4-(*E*)-desaturase, LCB Δ8-(*E*/*Z*)-desaturase] modifying the hydrophobic ceramide core belong to a large superfamily of membrane-bound proteins including fatty acid desaturases involved in the biosynthesis of polyunsaturated fatty acids. These oxygen-dependent enzymes are characterized by three conserved histidine motifs which may be involved in binding a di-iron complex [118]. A phylogram derived from amino acid alignments of these sphingolipid desaturases and hydroxylases shows four distinct branches originating in the middle of the phylogram which indicates a very early separation of these paralogous groups (Fig. 4). It is assumed that enzymes with identical or similar regioselectivity are

also similar in their amino acid sequence [118,128]. Sphingolipid Δ8-desaturases are more similar to fatty acyl Δ5- and Δ6-desaturases all of which are cytochrome b_5 fusion proteins [119,129] and have evolved independently of sphingolipid Δ4-desaturase activity [18]. Interestingly, the fatty acyl lipid Δ4-desaturase, which recently has been cloned from *Thraustochytrium* sp. and which is also a cytochrome b_5-fusion protein [130], is also more similar to the fatty acyl Δ5/Δ6-desaturases than to the non-fused sphingolipid Δ4-desaturase and C4-hydroxylase. Thus, Δ4/C4-regioselectivity must have evolved independently three times pointing to a convergent evolution of fatty acyl Δ4-desaturase, sphingolipid Δ4-desaturase and sphingolipid C4-hydroxylase. Interestingly, in mouse, a sphingolipid Δ4-desaturase paralogue has acquired additional C4-hydroxylase activity resulting in a bifunctional enzyme [16] which could be regarded as a "reinvention of the wheel". The invariant fusion between sphingolipid Δ8-

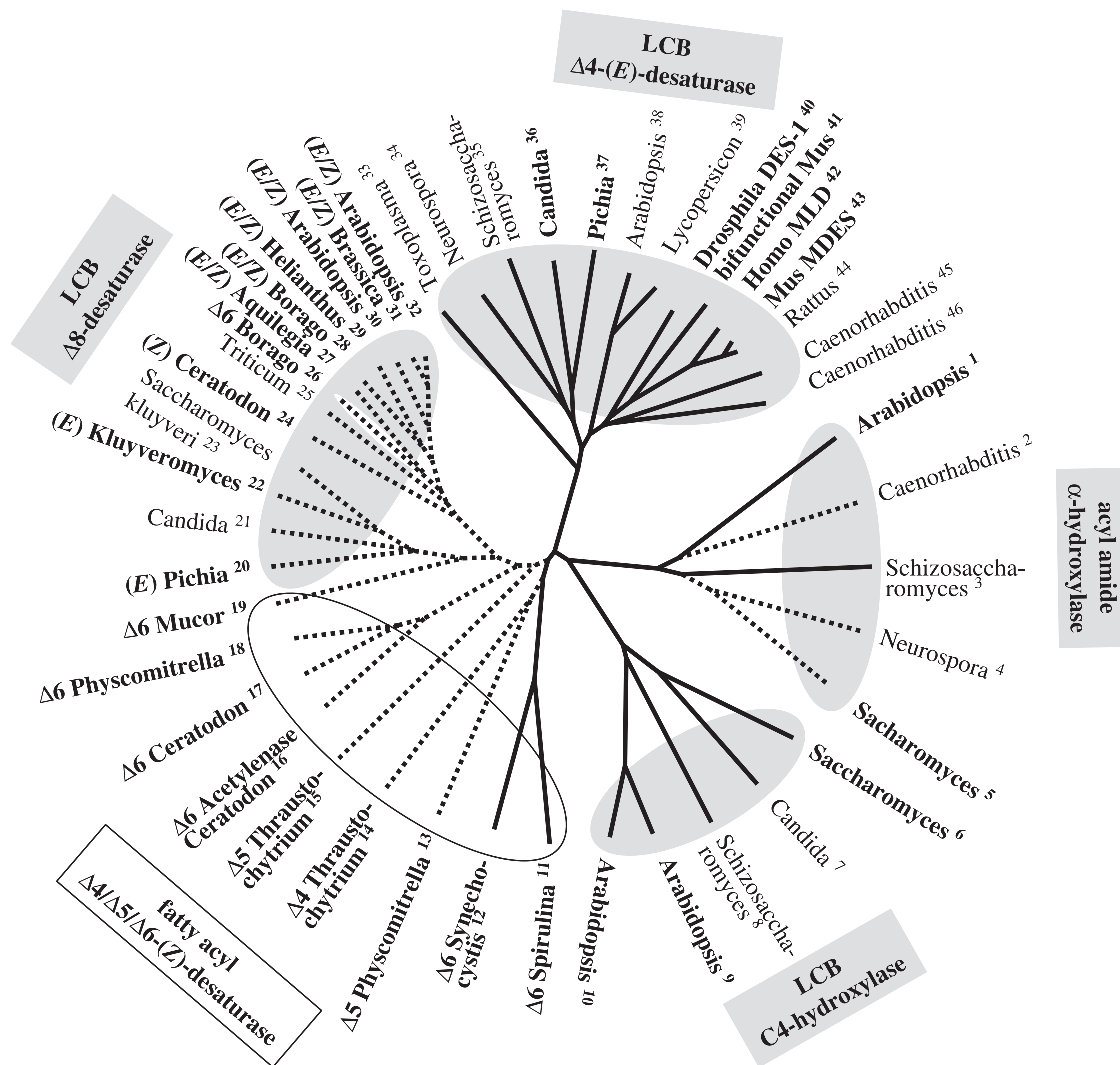


Fig. 4. Phylogram showing similarities between sphingolipid desaturases/hydroxylases and selected Δ4/Δ5/Δ6-fatty acyl desaturases. Full-length amino acid sequences were aligned and grouped in a radial diagram using the programs CLUSTALX and TreeView. The regioselectivities of lipid desaturases are indicated by Δ-numbers, their stereoselectivity by (*E*), (*Z*) or (*E*/*Z*) and the presence of a *N*-terminal cytochrome b_5 fusion domain [119] by dotted lines. All sphingolipid-modifying enzyme groups are marked by grey background. Note the close similarity of the Δ6-fatty acyl desaturases from *B. officinalis* and *Mucor rouxii* to the Δ8-LCB desaturases. For clarity, only a selection of available fatty acyl desaturase sequences has been included to construct this dendrogram. The inclusion of more presently known fatty acyl desaturase sequences does not affect the branchings of the sphingolipid-modifying enzymes [18]. Functionally identified enzymes are highlighted by bold lettering. Accession numbers: [1] AF02104, [2] Z81038, [3] Z97209, [4] CAD21081, [5] Z49260, [6] AAA16608, [7] *C. albicans* ORF 6.4041 on contig 6-2307, [8] CAA21900, [9] AC013289, [10] AC012188, [11] X87094, [12] L11421, [13] unpublished, [14] AF489589, [15] AF489588, [16] AJ250734, [17] AJ250735, [18] AJ222980, [19] AF296076, [20] unpublished, [21] *C. albicans* genomic sequence on contig 6-1607, bases 2181-430, [22] BAB93118, [23] BAB93117, [24] unpublished, [25] AF031194, [26] U79010, [27] AF406816, [28] AF133728, [29] X87143, [30] AJ224161, [31] AJ224160, [32] AC005397, [33] BAB58879, [34] *Neurospora crassa* genomic sequence on contig 9a58, bases 17916-16535, [35] T40333, [36] *C. albicans* genomic sequence on contig 6-2340, bases 7499-8611, [37] unpublished, [38] AAD17340, [39] AF466378, [40] AF466379, [41] AF466377, [42] AF466375, [43] AF466376, [44] NP_445775, [45] NP_501256, [46] NP_493549.

desaturases and cytochrome b_5 which represents the immediate electron donor for many microsomal desaturases, may have a functional advantage [119]. At present, we do not know why this fusion is only present occasionally in the acyl amide α-hydroxylases and absent in the sphingolipid Δ4-desaturase and C4-hydroxylase.

3.3. Formation of complex sphingolipids

One possible modification of the primary hydroxyl group of ceramides occurs by glycosylation yielding cerebrosides. cDNAs coding for UDP-glucose:ceramide β-D-glucosyltransferase (GCS) were isolated from *H. sapiens* [131] and some fungi [15,132,133], whereas no homologs are found in *S. cerevisiae* or *S. pombe* which is consistent with the lack of cerebrosides in these yeasts. Recently, also the first plant GCS has been identified from *Gossypium arboreum* (cotton) by its functional expression in *P. pastoris* [15] showing similarity to a putative GCS sequence (AF424585) in *A. thaliana*. There is only little information on the substrate specificity of plant GCS. Studies on the mammalian enzyme showed that it requires UDP-glucose and *N*-acyl-D-*erythro*-sphinganine and that it does not accept the L-*erythro* enantiomer or the L-*threo* diastereomer [134].

Unexpectedly, an in vitro assay using microsomal membranes from bean hypocotyls and radiolabelled sterol glucoside as substrate demonstrated UDP-glucose-independent GCS activity [135]. The assumption that sterol glucoside may function as a glucosyl donor is in line with recent data that sterol glucosides act as primers for cellulose synthesis in plants [82,136]. In contrast, a sterol glucosyltransferase/GCS double null-mutant of *P. pastoris* expressing the cotton GCS resulted in sterol glucoside-independent glucosylceramide synthesis, suggesting that UDP-glucose is the actual sugar donor [137]. The presence of a *N*-terminal transmembrane domain and the sterol glucoside-independency of the plant enzyme supports a location in the Golgi apparatus as suggested for the mammalian enzyme rather than an exoplasmic orientation in the plasma membrane [138].

Expression of the cotton GCS in a *P. pastoris* GCS-null mutant resulted in characteristic glucosylceramide species with non- and α-hydroxylated C16- to C24-acyl amides, suggesting that in fungi, initial Δ4-(*E*)-desaturation is followed by Δ8-(*E*)-desaturation and final C9-methylation of the LCB [15]. The fact that (4*E*,8*E*)-9-methyl-sphinga-4,8-dienine is the major LCB in free ceramides of several fungi supports the assumption that ceramide glycosylation succeeds LCB modifications [139]. However, it is not clear whether in plants, cerebroside formation occurs before or after the modifications of the hydrophobic ceramide core.

The formation of other complex plant sphingolipids such as mannosylceramide and GIPC is still unclear, because no gene coding for a mannosylceramide synthase or for an inositol phosphorylceramide synthase has been identified in plants yet. In *S. cerevisiae*, the first step in GIPC formation is catalyzed by the posphatidylinositol: ceramide phosphoinositol transferase (IPC synthase) which transfers the inositolphosphate moiety from phosphatidylinositol to the C-1 hydroxy group of ceramide [140]. The membrane-bound enzyme has been shown to be located in the Golgi apparatus of *S. cerevisiae* [141]. IPC synthase activity is inhibited by antifungal agents such as aureobasidin A [142–145], khafrefungin [146] and rustmycin [147]. Because the enzyme activity is essential [144,148], but missing in mammals, it represents a promising target for antifungal drugs. In *S. cerevisiae*, the IPC synthase or a subunit of the enzyme is encoded by the *AUR1* gene [142,144]. Homologs of the *AUR1* gene were identified from a number of human pathogenic fungi [149]. Surprisingly, a similar gene cannot be found in the *A. thaliana genome* or in other plant genomes such as rice, wheat, barley and oat. Due to the lack of any in vitro and in vivo studies or data concerning the effectiveness of the antifungal IPC synthase inhibitors in plant systems, there is no evidence for the existence of an IPC synthase in plants yet. Therefore, in plants, either a completely different (insensitive) protein or a different reaction mechanism may be involved in this step. At present, nothing is known about the steps of GIPC biosynthesis succeeding ceramide formation in plants representing an open field for molecular and biochemical investigations.

4. First functions of sphingolipids in plants

There is still little information on the functions of sphingolipids in plants. Their roles in cell signalling, membrane stability, stress response, pathogenesis and apoptosis have been recently reviewed [25–27]. However, there are interesting roles emerging which will be shortly summarized.

4.1. Cell signalling

Recent informations indicate that sphingolipids are important cellular mediators not only in animals and fungi, but also in plants. The knock-out of an *A. thaliana* gene coding for a protein accelerating in vitro (*E*)-sphing-4-enine transfer between membranes caused activation of cell death and defense-related genes [150]. Evidence for the presence of (*E*)-sphing-4-enine-1-phosphate in plants has been achieved showing its involvement in drought-induced signal transduction in guard cells linking the perception of abscisic acid to a reduction in turgor [151]. Furthermore, sphing-4-enine-1-phosphate promoting Ca^{2+}-mediated guard cell closure required the presence of the Δ4-double bond. Therefore, the low abundance of sphing-4-enine in plant sphingolipids [6] does not indicate its biological insignificance, but may point either to a selective incorporation into rare sphingolipids or to an exclusive occurrence in specific tissues or cell types, respectively. Interestingly,

the *D. melanogaster* DES and mammalian MLD genes encoding sphingolipid Δ4-desaturases [16] had previously been characterized in unrelated molecular and genetic studies [152,153] without knowing the true function of the protein. The human MLD gene was shown to modulate expression of the EGF (epidermal growth factor) receptor which stimulates (*E*)-sphing-4-enine-1-phosphate production [152]. In *des* mutant flies, cell cycle and spermatid differentiation are specifically blocked at the entry of the first meiotic division leading to male sterility [153]. Though functional expression of higher plant DES orthologs has failed so far, it would be of particular interest to see, whether plant Δ4-desaturated metabolites have similar signalling functions required in the sporophyte (meiosis) or gametophyte. In the filamentous fungus *Aspergillus nidulans*, gene disruption was used to inactivate the inositol phosphorylceramide synthase (*aurA*) and the serine palmitoyltransferase (*lcb2*), respectively. The resulting phenotypes indicated that sphingolipids are essential for the establishment and maintenance of cell polarity via control of the actin cytoskeleton and that accumulation of ceramide is likely responsible for arresting the cell cycle in G_1 [148]. Future investigations of *A. thaliana* mutants will show whether certain sphingolipid metabolites play a similar important role in controlling cell polarity and cell cycle progression in plants.

4.2. Membrane stability

Sphingolipids are also important for membrane organization. Mammalian and fungal sphingolipids have a tendency to associate with cholesterol or ergosterol, respectively, and form clusters of raft-like domains, which are important for lateral sorting of proteins, cellular trafficking and signal transduction [154–156]. Many cell surface proteins interact with such lipid rafts through their transmembrane domains or lipid anchors such as saturated acyl chains or glycosylphosphatidylinositol/glycosylphosphorylinositol ceramides (GPI) [73,157]. There is new evidence from detergent insolubility that plasma membrane microdomains exist in plant cells as well, although they contain sterols other than cholesterol [158]. The plant-specific sterols sitosterol and stigmasterol, differing in aliphatic side chain structure from cholesterol, by an additional ethyl group at C-24 and the latter by an additional Δ22-(*E*)-double bond, also promote domain formation, which is modulated by sterol side chain structure [159]. Moreover, small amounts of free ceramide significantly stabilize domain formation, suggesting that this signaling molecule is likely to concentrate within sphingolipid/sterol rafts. The presence of GPI-anchored proteins in plants has been discovered only recently [74–78], thereby leading to the identification of a *COB(RA)*-like multigene family in *A. thaliana* encoding putative GPI-anchored proteins, which are likely to be involved in orientated cell expansion at the plasma membrane–cell wall interface of vascular plants [79].

4.3. Abiotic stress response

Sphingolipids have been implicated in conferring stability to plant membranes, contributing to acclimation to drought stress [84] and to cold hardiness in chilling-resistant plants [6,7,59,86,87,160,161]. It has been observed that the proportion of glucosylceramides in plasma membranes of freezing-tolerant plants is lower than in freezing-sensitive plants and that the glucosylceramide content is reduced following cold acclimation [86,87,160,161]. The molecular species composition of cerebrosides differed among chilling-sensitive and -tolerant plants and changed during cold acclimation. Among many plant species analysed, hydroxy nervonic acid (24h:1) was only found in the leaf cerebrosides of chilling-resistant plants, suggesting that cerebroside species with monounsaturated very long-chain hydroxy acyl amides exhibit much lower phase transitions than those having a saturated hydroxy acyl amide residue [56,59]. Furthermore, most of the chilling-resistant plant species analysed had more 8-(*Z*)-unsaturated than 8-(*E*)-trihydroxybases, suggesting that high levels of (8*E/Z*)-4-hydroxysphing-8-enine are correlated with freezing tolerance [6,7].

4.4. Phytopathogenesis

A new field of sphingolipid functions comprises plant–pathogen interactions. Previous reports have shown that certain cerebrosides isolated from fungi [162–165], annelida [166] and plants [167] stimulate fruiting body formation of *Schizophyllum commune*, a fungus involved in wood degradation. The active glucosylceramides from wheat grain consisted of α-hydroxylated C_{16}- or C_{18}-acyl amides, and of (4*E*,8*Z*)-sphinga-4,8-dienine or (*Z*)-sphinga-8-enine [167]. Hydrogenation of the (*Z*)-sphinga-8-enine-containing glucosylceramide showed that the stimulatory effect of these cerebrosides was dependent on the presence of a Δ8-double bond. More recent studies showed that fungal cerebrosides function as elicitors causing hypersensitive cell death, phytoalexin accumulation and increased resistance to subsequent infections by compatible pathogens in plants [168–170]. Elicitor-active glucosylceramides were isolated from the rice pathogenic fungus *Magnaporthe grisea* having an amide-linked (3*E*)-2-hydroxyhexadec-3-enoyl or (3*E*)-2-hydroxyoctadec-3-enoyl group bound to (4*E*,8*E*)-9-methyl-sphinga-4,8-dienine [168]. Interestingly, hydrogenation of the Δ8-(*E*)-double bond in the LCB or of the Δ3-(*E*)-double bond in the acyl amide moiety of the cerebroside did not alter elicitor activity, whereas the Δ4-(*E*)-double bond of the LCB and the methyl group at C-9 were essential for elicitor activity [168]. The glucose headgroup was not crucial, because free ceramide also showed elicitor activity though with reduced effectiveness [169]. In field experiments with application of as little as 3×45 g/ha, these glucosylceramide elicitors, which occur in many different phytopathogens, protected

rice plants against *M. grisea* and other diseases as well, indicating that cerebrosides function as general elicitors in a wide range of rice–pathogen interactions [169,170]. The importance of the Δ8-double bond in fungal fruiting body induction or of the Δ4-double bond and 9-methyl group in the hypersensitive response to phytopathogens indicates that diverse structural LCB modifications are contributing to different cellular responses in plant–pathogen interactions. The observation of an increase in the expression of serine palmitoyltransferase during the hypersensitive response of a late-blight-resistant potato to *Phytophthora infestans* [171] also points to the involvement of sphingolipids in pathogenesis.

Further evidence for the involvement of sphingolipids in this process was provided by showing that the *IPT1* gene encoding the mannose-(inositol-phosphate)$_2$-ceramide synthase determines the sensitivity of *S. cerevisiae* to an antifungal plant defensin [172]. In contrast to the sensitive wild-type strain, an *ipt1* yeast mutant became highly resistant to dahlia defensin-mediated plasma membrane permeabilization [172]. The data support a model in which lipid rafts containing GIPC may act as binding sites for plant defensins or are required to anchor associated membrane proteins, which in turn interact with these antimicrobial peptides. The interaction with these docking sites would enable plant defensins to insert into the plasma membrane of fungal hyphae leading to membrane permeabilization and reduced hyphal elongation.

4.5. Programmed cell death

Evidence for the involvement of sphingolipids in apoptosis and ceramide signalling in plants comes from investigations of sphinganine-analogous mycotoxins (SAMs) such as fumonisins produced by *Fusarium moniliforme* and related fungi, and host-selective toxins secreted from *Alternaria alternata* f sp. *Lycopersici* (AAL), which have been shown to be cytotoxic and cancerogenic in animals [173–175]. SAMs have been shown to induce necrosis, DNA fragmentation and accumulation of free LCB in different plant tissues [176–179], which could be attributed to a competitive inhibition of the sphinganine *N*-acyltransferase (ceramide synthase) activity [5]. In tomato, codominant insensivity to SAMs is mediated by the *ASC1* gene (*Alternaria* stem canker), which is homologous to the yeast longevity assurance gene (*LAG1*) and facilitates the ER-to-Golgi transport of GPI-anchored proteins [100]. Overexpression of Asc1 in SAM-sensitive plants resulted in resistance to infection by *A. alternata* f. sp. *lycopersici*, indicating that susceptibility of tomatoes for SAMs may involve sphingolipids and ER-to-Golgi transport of GPI-anchored proteins. Labelling experiments of Asc/Asc and asc/asc tomato leaf discs with tritiated serine indicated that the presence of Asc1 is able to relieve an AAL toxin-induced block of sphingolipid synthesis that otherwise would lead to programmed cell death [180]. Today, natural and synthetic inhibitors are known covering nearly all steps of sphingolipid synthesis and metabolism [105,147,173] which could be useful for inhibitor experiments leading to substrate accumulation and product impoverishment to investigate the function of specific sphingolipid metabolites. Taken together, the emerging data on fruiting body induction, elicitor function and the effects of mycotoxins emphasize the role of plant sphingolipids in plant–pathogen interactions.

5. Conclusions

Since the complete genomic sequence of *A. thaliana* is available and sequencing projects of other plant genomes are currently in progress, putative candidate genes coding for nearly every enzyme involved in plant sphingolipid metabolism may be identified in databases. After annotation, genes can be functionally characterized by heterologous expression in suitable eukaryotic model organisms such as yeast and *S. pombe*, or by complementation of orthologous mutants. Subsequently, further enzymatic characterization, elucidation of substrate specificities and actual sequence of enzymatic steps as well as subcellular localization of proteins and promotor studies, are urgently needed. The generation of homozygous *A. thaliana* mutants and of subsequent suppressor mutants will enable a new approach to study sphingolipid functions in plants, which will include the role of sphingolipids in cell–cell interactions and in stress response. Plants recognize fungal cerebrosides and in response activate several defense mechanisms, despite the fact that cells of both organisms are surrounded by cell walls not present in animal tissues. Future studies will investigate the mechanism of plant–pathogen relationships and will show, which structural features of sphingolipids are involved in this crosstalk between plants and fungi.

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