# Chapter 5

# Plant Sphingolipids: Structure, Synthesis and Function

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*H. Wada and N. Murata (eds.), Lipids in Photosynthesis: Essential and Regulatory Functions, pp. 77–115.*

## Summary

Sphingolipids are major structural components of endomembranes and dynamic regulators of basic cellular processes in plants. Advances during the past decade have revealed that sphingolipids are essential molecules in plants, and many of the genes for sphingolipid biosynthetic enzymes have been identified and characterized. In addition, improved methods for sphingolipid extraction and analysis have uncovered the immense structural complexity and quantitative importance of sphingolipids in plant cells. These advanced analytical methods have also been increasingly applied to the characterization of *Arabidopsis thaliana* mutants to provide unexpected insights into sphingolipid metabolism and function. Complementing these studies is a growing awareness that sphingolipids are one of the most abundant lipid components of the plasma membrane of plant cells and may play a role in the organization and function of membrane microdomains that are important for cell surface activities and for trafficking of proteins to the plasma membrane. Furthermore, sphingolipid metabolites including free and phosphorylated forms of long-chain bases and ceramides have been linked as bioactive regulators to a number of cellular processes (e.g., programmed cell death) that are important for abiotic stress resistance, plant development, and plant–pathogen interactions. This review provides a synopsis of the rapidly progressing field of plant sphingolipid biology and highlights gaps in our knowledge of the metabolism and function of these molecules in plants.

*Abbreviations:* ABA – Abscisic acid; acd – Accelerated cell death; ACP – Acyl carrier protein; AAL – *Alternaria alternata* f. sp. *lycopersici;* GBA – Bile acid β-glucosidase; DRM – Detergent-resistant membranes; ER – Endoplasmic reticulum; FATB – Fatty acid thioesterase B; GlcCer – Glucosylceramide; GLTP – Glycolipid transfer protein; GIPC – Glycosyl inositolphosphoceramide; HR – Hypersensitive response; IPC – Inositolphosphoceramide; $L_d$ – Liquid-disordered phase; $L_o$ – Liquid-ordered phase; LCB – Long-chain base; LCB-P – Long-chain base-1-phosphate; $T_m$ – Melting temperature; PR – Pathogenesis-related; PI – Phosphatidylinositol; RNAi – RNA interference; SPT – Serine palmitoyltransferase; d18:0 – Sphinganine (dihydro sphingosine); SPHK – Sphingosine kinase; VLCFA – Very long-chain fatty acid; t18:0 – 4-Hydroxysphinganine (phytosphingosine); t18:1Δ8*trans* – 4-Hydroxy-Δ8*trans*-sphingenine; t18:1Δ8*cis* – 4-Hydroxy-Δ8*cis*-sphingenine; d18:1Δ4*trans* – Δ4*trans*-sphingenine (sphingosine); d18:1Δ8*trans* – Δ8*trans*-sphingenine; 18:1Δ8*cis* – Δ8*cis*-sphingenine; d18:2Δ4*trans*,8*cis* – Δ4*trans*,8*cis*-sphingadienine; d18:2Δ4*trans*,8*trans* – Δ4*trans*,8*trans*-sphingadienine

## I Introduction

Sphingolipids are essential components of the endomembrane system in plants and other eukaryotes. Following their discovery in plants in the 1950s (Carter et al., 1958a, b), sphingolipids received only modest study due, in part, to the challenges associated with their analyses and the assay of sphingolipid metabolic activities in plant extracts. The quantitative significance of sphingolipids in plant cells was also not fully appreciated. However, sphingolipid biology has become an emerging area of plant lipid research during the past 5–10 years. The advances in sphingolipid research in plants have been guided by increased knowledge of sphingolipid metabolism in yeast and mammals (Lynch and Dunn, 2004). In addition, tools have been developed that make sphingolipid research in plants more tractable. These include functional genomic tools,

particularly collections of *Arabidopsis thaliana* insertion mutants that have enabled researchers to examine the consequences of altered gene expression on sphingolipid metabolism and function (e.g., Chen et al., 2006, 2008; Tsegaye et al., 2007; Dietrich et al., 2008). In addition, refined sphingolipid analytical protocols have been developed that allow for quantitative extraction of sphingolipids and comprehensive structural profiling of sphingolipids from plant tissues (Markham et al., 2006; Markham and Jaworski, 2007). Furthermore, an increasing number of forward genetic studies of mutants affected in plant development and pathogen resistance have uncovered new and unexpected roles of sphingolipids in plant biology (e.g., Koga et al., 1998; Stone et al., 2000; Brandwagt et al., 2002; Liang et al., 2003; Shi et al., 2007).

It is now recognized that sphingolipid function in plants is multi-faceted. Sphingolipids are primarily major structural components of endomembranes and have been estimated to compose ≥40% of the lipids of the plasma membrane and are also enriched in tonoplast (Verhoek et al., 1983; Yoshida and Uemura, 1986; Haschke et al., 1990; Sperling et al., 2005; Laloi et al., 2007). In addition to providing structural integrity to membranes, growing evidence supports a role of sphingolipids in detergent-resistant membrane (DRM) fractions or lipid rafts that are important for organization and function of proteins on the surface of plant cells and for trafficking of proteins through the Golgi to the plasma membrane (Mongrand et al., 2004; Borner et al., 2005; Laloi et al., 2007; Lefebvre et al., 2007). In addition, sphingolipids can function through biosynthetic intermediates and metabolites to mediate cellular processes, such as programmed cell death and ABA (abscisic acid)-dependent signal transduction (Ng et al., 2001; Coursol et al., 2003; Worrall et al., 2003).

Underlying the function of sphingolipids in plants is an intricate biosynthetic pathway that uses fatty acid and amino acid precursors to generate a wide array of sphingolipid structures and involves membrane-associated reactions in multiple subcellular compartments. The hundreds of different sphingolipid molecules that arise from this pathway have distinct physical and biochemical properties that can impact their functions in plant cells. Sphingolipid synthesis in plants is also balanced by catabolic pathways that not only provide a mechanism for the turnover of sphingolipids but can also generate bioactive molecules that regulate diverse cellular processes (Ng et al., 2001; Coursol et al., 2003; Liang et al., 2003).

In this review, we highlight current knowledge of sphingolipid structure, metabolism, and function in plants. We also describe gaps in our knowledge of sphingolipid biology that will likely be topics for future study in this rapidly evolving area of plant lipidology.

## II Sphingolipid Structure

Like membrane glycerolipids, sphingolipids are amphiphilic molecules that contain a polar head group and two hydrophobic acyl chains (Fig. 1). The hydrophobic portion of sphingolipids is contained in their ceramide backbone, which is composed of a fatty acid bound through an amide linkage to a long-chain base (LCB). Fatty acids of plant sphingolipids typically range in chain length from 16 to 26 carbon atoms and are either saturated or monounsaturated with a *cis*-ω9 double bond (Imai et al., 2000). Small amounts of very long-chain fatty acids (VLCFAs) with odd numbers of carbon atoms (e.g., C21, C23, and C25) are also detectable in sphingolipids (Sastry and Kates, 1964; Carter and Koob, 1969). In addition, the fatty acid moiety frequently occurs with a α- (or C-2-) hydroxy group (Sastry and Kates, 1964; Carter and Koob, 1969).

The LCB is a unique component of sphingolipids and sphingolipid metabolites that is a combination of an amino acid (serine) and a fatty acid (typically palmitic acid, 16:0) (Fig. 2). In plants, LCBs contain 18 carbon atoms and are characterized by the presence of either two or three hydroxyl groups. Dihydroxy LCBs contain hydroxyl groups at the C-1 and C-3 positions, while trihydroxy LCBs contain an additional hydroxyl group at the C-4 position. Dihydroxy LCBs and trihydroxy LCBs occur in planta in the D-*erythro* and D-*ribo* configurations, respectively. The initial LCB produced in plants is sphinganine (or dihydrosphingosine), which is fully saturated and contains two hydroxyl groups.

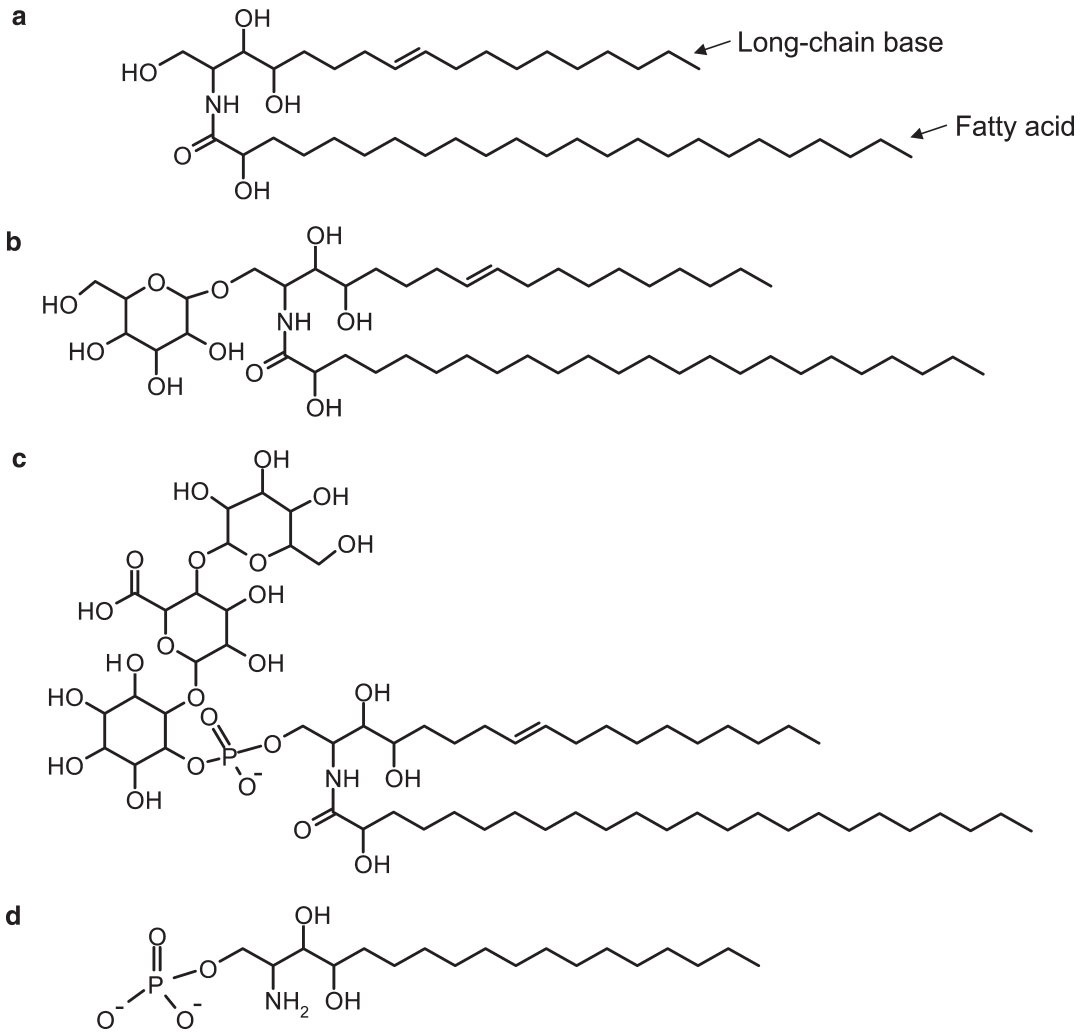


*Fig. 1.* Examples of complex sphingolipids and sphingolipid metabolites found in plants. Complex sphingolipids consist of a polar head group bound to a hydrophobic ceramide **(a)**. The ceramide backbone comprises a fatty acid bound to a C18 long-chain base through an amide linkage. The two major classes of complex sphingolipids in plants are glucosylceramides (GlcCers) and glycosyl inositolphosphoceramides (GIPCs), examples of which are shown in **(b)** and **(c)**. GlcCers contain a glucose bound through a 1,4 glycosidic linkage to a ceramide **(b)**. The GIPC shown in (**c**), a hexose-hexuronic acid-inositolphosphoceramide, is the major GIPC in *Arabidopsis thaliana*. Shown in **(d)** is a long-chain base-1-phosphate (LCB-P). Certain structural forms of LCB-Ps are believed to be involved in the regulation of cellular processes, such as ABA-dependent stomatal closure.

This LCB can be further modified by the addition of not only a C-4 hydroxyl group, but also by introduction of double bonds between the C-4 and C-5 atoms and the C-8 and C-9 atoms. The Δ4 double bond occurs exclusively in the *trans* configuration, whereas the Δ8 double bond can be either *cis* or *trans*. In contrast to mammals, Δ4-monounsaturated LCBs (Δ4*trans*-sphingenine or sphingosine) are typically of low abundance in plant sphingolipids. Instead, the Δ4 double bond is more frequently found in diunsaturated LCBs in combination with a Δ8 double bond (Lynch and Dunn, 2004). The most widely occurring LCBs in plant sphingolipids are sphinganine (or dihydrosphingosine; d18:0), 4-hydroxysphinganine (or phytosphingosine; t18:0), 4-hydroxy-Δ8*cis/trans*-sphingenine (Δ8*cis*- or *trans*-t18:1), and sphingadiene (Δ4*trans*-, Δ8*cis*- or *trans*-d18:2) (Lynch and Dunn, 2004) (Fig. 2). The relative amounts of these LCBs in ceramides of sphin-

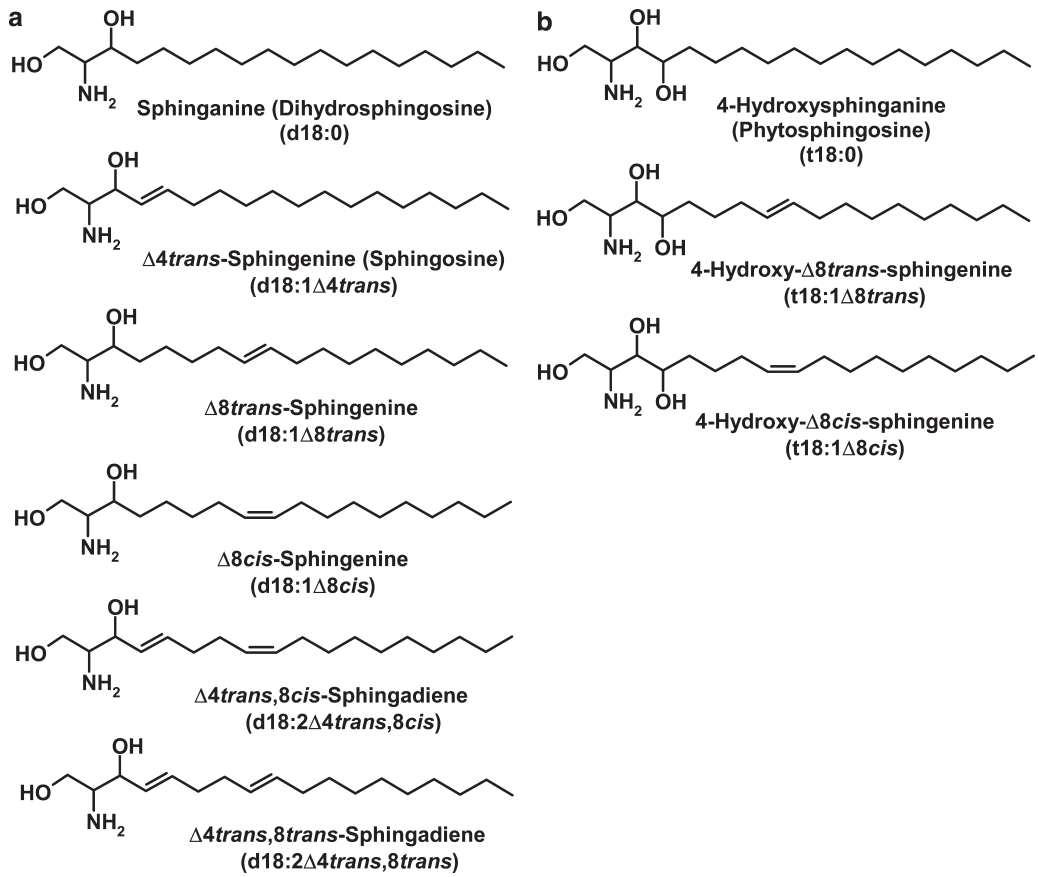


*Fig. 2.* Long-chain bases (LCBs) found in plant sphingolipids. Plant LCBs are derived from the condensation of serine and palmitoyl-CoA. **(a)** Dihydroxy LCBs contain hydroxyl groups at the C-1 and C-3 positions. **(b)** Trihydroxy LCBs contain an additional hydroxyl group at the C-4 position. Dihydroxy and trihydroxy LCBs can contain double bonds at the Δ8 position that are either in the *cis* or *trans* configurations. Dihydoxy LCBs can also contain a *trans* double bond at the Δ4 position. In the nomenclature used, d18:1Δ8*trans*, for example, indicates that the LCB is dihydroxy ("d") and contains 18 carbon atoms and one double bond at the Δ8 position that is in *trans* configuration. The "t" in the LCB names in **(b)** indicates that these molecules are trihydroxy LCBs.

golipids can vary widely among different organs of a single species and between different species. Although the vast majority of LCBs are found in ceramides, a small but detectable amount is present in plant cells in a free form or as a phosphate ester (Markham and Jaworski, 2007). The latter consist of a phosphate group bound to the C-1 hydroxyl of the LCB. LCB-1-phosphates (LCB-Ps) have been ascribed a number of bioactive properties in plants and other eukaryotes (e.g., Zhang et al., 1991; Dickson et al., 1997; Worrall et al., 2003; Taha et al., 2006) (Fig. 1).

The hydrophilic portion of sphingolipids consists of a polar head group that is bound to the C-1 atom of the LCB moiety of the ceramide. A large array of different head groups can be found in eukaryotes. These include carbohydrate residues that can be as simple as a glucose or galactose residue found in glucosyl- and galactosylceramides or as complicated as the extensive chains of sugar residues found in gangliosides. Other head groups include phosphocholine that is found in sphingomyelin and phosphoinositol-based moieties that are characteristic of yeast sphingolipids. To date, the two major classes of complex sphingolipids identified in plants are glucosylceramides (GlcCers) or glucocerebrosides and glycosyl inositolphosphoceramides (GIPCs) (Carter et al., 1958b, 1960; Kaul and Lester, 1975; Markham et al., 2006) (Fig. 1). The glucose residue in glucosylceramides

(GlcCers) is bound to the ceramide backbone in a 1,4 glycosidic linkage. GlcCers, together with free ceramides, are collectively referred to as neutral sphingolipids. The GIPCs, by contrast, are charged sphingolipids that contain a phosphoinositol group attached to the C-1 hydroxyl of the ceramide backbone through a phosphoester linkage (Carter et al., 1958b; Kaul and Lester, 1975). The inositol residue is substituted with additional sugar or sugar-derived residues. The major GIPC of *Arabidopsis thaliana*, for example, was recently identified as hexose-hexuronic acid-inositolphosphoceramide (Markham et al., 2006). The occurrence of even more complex GIPCs has been reported in tobacco. These include GIPCs with head groups containing two arabinose and two galactose moieties and two arabinose, two galactose, and one mannose moieties (Kaul and Lester, 1978).

Of the two major classes of sphingolipids, GlcCers have been more extensively characterized in plants. This is largely due to their ease of purification using traditional methods for extraction of plant lipids. GlcCers can also be enriched in lipid extracts based on the resistance of the amide bond between the fatty acid and LCB to mild alkaline hydrolysis. Similar treatment readily releases fatty acids from the glycerol backbone of the more abundant glycerolipids. GIPCs, however, are largely non-recoverable using the common chloroform/methanol-type extraction methods because of the high degree of polarity associated with their head groups. Recently, yeast lipid extraction protocols that use more polar solvents and heating have been adapted for the quantitative extraction of sphingolipids from plant leaves (Markham et al., 2006). Through the use of these protocols, it was recently determined that GIPCs are the most abundant class of sphingolipids in *Arabidopsis thaliana* leaves, accounting for approximately 60–65% of the total sphingolipids (Markham et al., 2006). GlcCers, by comparison, make up about 30% of the total sphingolipids in *Arabidopsis thaliana* leaves. In addition to GIPCs and GlcCers, ceramides, free LCBs, and LCB-Ps account for <10% of the total sphingolipid fraction in *Arabidopsis thaliana* leaves. It is also notable that phosphoceramides have been reported as anchors of arabinogalactan proteins in plasma membrane of pear and rose (Oxley and Bacic, 1999; Svetek et al., 1999). This is similar to the role that glycosylphosphatidylinositol (GPI) plays as a membrane anchor of proteins in plants and other eukaryotes (Paulick and Bertozzi, 2008). The quantitative significance of phosphoceramides as protein anchors in plants has yet to be determined (Oxley and Bacic, 1999; Svetek et al., 1999).

## III Synthesis of Sphingolipids

### *A Ceramide Synthesis*

#### *1 Serine Palmitoyltransferase*

The LCB component of ceramides results from the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine (Fig. 3). This reaction is catalyzed by serine palmitoyltransferase (SPT), a member of the pyridoxal phosphate-dependent α-oxamine synthase subfamily. The *Arabidopsis thaliana* SPT has been shown to consist of LCB1 and LCB2 subunits, which is similar to the subunit structure of other eukaryotic SPTs (Tamura et al., 2001; Chen et al., 2006; Dietrich et al., 2008). Although both LCB1 and LCB2 share structural similarity to α-oxamine synthases, the catalytic lysine residue that forms a Schiff base with pyridoxal phosphate resides in the LCB2 subunit (Tamura et al., 2001). Based on studies of the *Saccharomyces cerevisiae* enzyme, it is believed that the active site of SPT is at the interface of LCB1 and LCB2, and LCB1 functions to stabilize LCB2 (Gable et al., 2002; Han et al., 2004). SPT is generally regarded as the primary regulated step in sphingolipid biosynthesis in yeast and mammals, with regulation occurring at both the transcriptional and post-transcriptional levels (Hanada, 2003). Detailed studies on the biochemical properties and regulation of the plant SPT have yet to be reported. Studies of knock-out mutants for the LCB2 subunit of the *Arabidopsis thaliana* SPT have shown unequivocally that sphingolipid synthesis is essential for the viability of plant cells (Dietrich et al., 2008; Teng et al., 2008). The *Arabidopsis thaliana* LCB2 is encoded by two genes designated *LCB2a* (At5g23670; Table 1) and *LCB2b* (At3g48780) that are constitutively expressed and encode redundant polypeptides (Dietrich et al., 2008). Mutants containing a homozygous knock-out of one gene and a hetero-

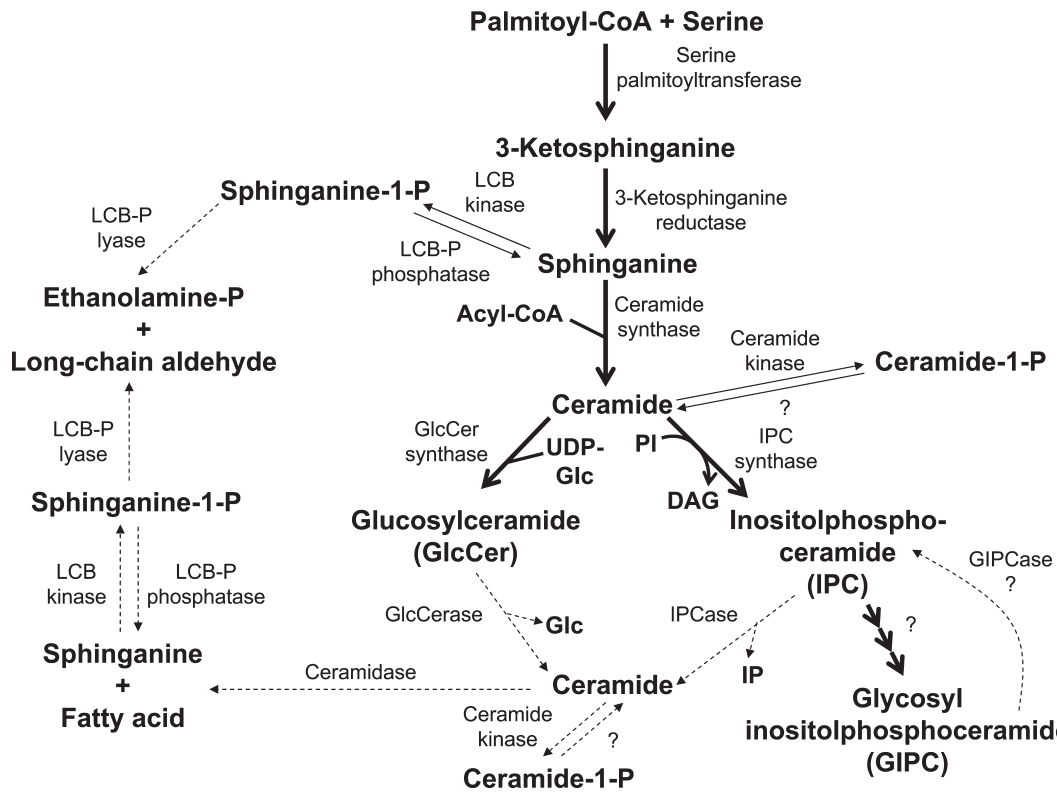


*Fig. 3.* Integrated pathways for the biosynthesis and turnover of sphingolipids in plants. *Solid arrows* indicate biosynthetic steps and dashed arrows indicate catabolic steps. *Arrows labeled with "?"* correspond to reactions that have yet to be identified in plants. Abbreviations: GlcCer, glucosylceramide; Glc, glucose; PI, phosphatidylinositol; DAG, diacylglycerol; IP, inositolphosphate; GlcCerase, glucosylceramidase; GIPC, glycosyl inositolphosphoceramide; GIPCase, glycosyl inositolphosphoceramidase; IPCase, inositolphosphoceramidase; LCB, long-chain base; LCB-P, long-chain base-1-phosphate; Ceramide-1-P, ceramide-1-phosphate.

zygous knockout for the second gene contain 50% aborted pollen, consistent with male gametophytic lethality resulting from loss of sphingolipid synthesis (Dietrich et al., 2008). In addition, partial RNAi suppression of the one *LCB1* gene (At4g36480; Table 1) of *Arabidopsis thaliana* was accompanied by reduced growth (Chen et al., 2006). However, the sphingolipid LCB content of these plants on a dry weight basis was unaffected, suggesting that plants compensate for down-regulation of sphingolipid synthesis by reduced growth (Chen et al., 2006).

### *2 3-Ketosphinganine Reductase*

In the second step of LCB synthesis, 3-ketosphinganine, the product of SPT, is reduced by the enzyme 3-ketosphinganine reductase to form sphinganine (d18:0), the simplest LCB found in plants (Fig. 3). 3-Ketosphinganine reductase is encoded by two genes in *Arabidopsis thaliana* (At3g06060, *KSR-1* and At5g19200, *KSR-2*; Table 1). Although both genes contribute to the reductase activity and are essential, *KSR-1* is more highly expressed in *Arabidopsis thaliana* leaves and is the major contributor to in vivo sphinganine production (M. Chen and E.B. Cahoon, unpublished data). The saturated dihydroxy LCB d18:0 resulting from the combined activities of SPT and 3-ketosphinganine reductase is available for incorporation into ceramides. However, in plant sphingolipids, d18:0 is typically a minor LCB (Sperling et al., 2005; Markham et al., 2006). Instead, the majority of the d18:0 undergoes C-4 hydroxylation to form the trihydroxy LCB t18:0, a reaction that most likely occurs prior to incorporation of the LCB into ceramides. LCB C-4 hydroxylation is described in more detail below.

*Table 1.* Putative and characterized *Arabidopsis* genes involved in sphingolipid metabolism.

| *Arabidopsis* gene (AGI code) | Designated gene symbol | Function (characterized or predicted) | *Saccharomyces cerevisiae* or mammalian homolog | References |
|---|---|---|---|---|
| At4g36480 | *LCB1* | Subunit of serine palmitoyltransferase | *LCB1* | Chen et al., 2006 |
| At5g23670 | *LCB2a* | Subunit of serine palmitoyltransferase | *LCB2* | Tamura et al., 2001; Dietrich et al., 2008 |
| At3g48780 | *LCB2b* | | | |
| At3g06060 | *KSR1* | 3-Ketosphinganine reductase | *TSC10* | Beeler et al., 1998; Dunn et al., 2004 |
| At5g19200 | *KSR2* | | | |
| At1g69640 | *SBH1* | LCB C-4 hydroxylase | *SUR2/SYR2* | Haak et al., 1997; Grilley et al., 1998; Chen et al., 2008 |
| At1g14290 | *SBH2* | | | |
| At3g61580 | *SLD1* | LCB Δ8 desaturase | None | Sperling et al., 1998; Ryan et al., 2007 |
| At2g46210 | *SLD2* | | | |
| At2g34770 | *FAH1* | Fatty acid α-hydroxylase | *FAH1/SCS7* | Haak et al., 1997; Mitchell and Martin, 1997 |
| At4g20870 | *FAH2* | | | |
| At4g04930 | None | LCB Δ4 desaturase | *DES1*, *DES2* | Ternes et al., 2002; Michaelson et al., 2009 |
| At3g25540 | *LOH1* | Ceramide synthase | *LAC*, *LAG1* | Brandwagt et al., 2000; Spassieva et al., 2002 |
| At3g19260 | *LOH2* | | | |
| At1g13580 | *LOH3* | | | |
| At2g37940 | *IPCS1* | IPC synthase | *IPCS*[a], *SMS1* | Yang et al., 2005; Denny et al., 2006 |
| At3g54020 | *IPCS2* | | | |
| At2g29525 | *IPCS3* | | | |
| At2g19880 | *GCS* | Glucosylceramide synthase | *GCS* | Leipelt et al., 2001 |
| At5g51290 | *ACD5/AtCERK* | Ceramide kinase | *LCB4*, *LCB5* | Liang et al., 2003 |
| At5g23450 | *AtLCBK1* | LCB kinase | | Imai and Nishiura, 2005 |
| At4g21540 | *SPHK1* | LCB kinase | | Worrall et al., 2008 |
| At2g46090 | *SPHK2* | Putative LCB kinase | | |
| At1g27980 | *AtDPL1* | LCB-1-phosphate lyase | *DPL1* | Tsegaye et al., 2007; Worrall et al., 2008 |
| At3g58490 | *LCB-PP1* | LCB-1-phosphate phosphatase | *LCB3/YSR*, *YSR3* | Worrall et al., 2008 |
| At5g03080 | *LCB-PP2* | | | |
| At4g22330 | *AtCES1* | Putative alkaline ceramidase | *YPC1*, *YDC1* | Mao et al., 2000a, b; Lynch and Dunn, 2004 |
| At1g07380 | None | Putative neutral ceramidase | *ASAH2* | Pata et al., 2008 |
| At2g38010 | None | | | |
| At5g58980 | None | | | |
| At5g49900 | None | Putative glucosylceramidase | *GBA2* | Boot et al., 2007 |

| | | | | |
|---|---|---|---|---|
| At1g33700 | None | | | |
| At4g10060 | None | | | |
| At3g24180 | None | | | |
| At4g29680 | None | Putative inositolphosphoceramidase | *ENPP7* | Duan et al., 2003a, b |
| At4g29690 | None | | | |
| At4g29700 | None | | | |
| At3g06460 | *AtELOs* | Putative condensing enzyme for very long-chain fatty acid synthesis | *ELO1*, *ELO2*, *ELO3* | Lynch and Dunn, 2004 |
| At3g06470 | | | | |
| At1g75000 | | | | |
| At4g36830 | | | | |
| At1g01120 | *KCS1* | 3-Ketoacyl-CoA synthase | None | Todd et al., 1999 |
| At1g04220 | *KCS2* | | | Paul et al., 2006 |
| At1g07720 | *KCS3* | | | Blacklock and Jaworski, 2006 |
| At1g19440 | *KCS4* | | | Blacklock and Jaworski, 2006 |
| At1g25450 | *KCS5/CER60* | | | Fiebig et al., 2000; Costaglioli et al., 2005 |
| At1g68530 | *KCS6/CER6/CUT1* | | | Fiebig et al., 2000; Costaglioli et al., 2005 |
| At1g71160 | *KCS7* | | | Blacklock and Jaworski, 2006 |
| At2g15090 | *KCS8* | | | Joubes et al., 2008 |
| At2g16280 | *KCS9* | | | Paul et al., 2006 |
| At2g26250 | *KCS10/FDH* | | | Yephremov et al., 1999 |
| At2g26640 | *KCS11* | | | Blacklock and Jaworski, 2006 |
| At2g28630 | *KCS12* | | | Joubes et al., 2008 |
| At2g46720 | *KCS13/HIC* | | | Gray et al., 2000; Costaglioli et al., 2005 |
| At3g10280 | *KCS14* | | | Joubes et al., 2008 |
| At3g52160 | *KCS15* | | | Joubes et al., 2008 |
| At4g34250 | *KCS16* | | | Blacklock and Jaworski, 2006 |
| At4g34510 | *KCS17* | | | Trenkamp et al., 2004 |
| At4g34520 | *KCS18* | | | Joubes et al., 2008 |
| At5g04530 | *KCS19/FAE1* | | | Paul et al., 2006 |
| At5g43760 | *KCS20* | | | Paul et al., 2006 |
| At5g49070 | *KCS21* | | | Joubes et al., 2008 |
| At1g67730 | *KCR* | 3-Ketoacyl-CoA reductase | *YBR159* | Beaudoin et al., 2002 |
| At1g24470 | *KCR*? | | | |
| At5g10480 | *HCD*, *PAS2* | 3-Hydroxyacyl-CoA dehydratase | *PHS1* | Bach et al., 2008 |
| At5g59770 | *HCD*? | | | |
| At3g55360 | *ECR*, *CER10* | Enoyl-CoA reductase | *TSC13* | Gable et al., 2004; Zheng et al., 2005 |

[a] Inositolphosphoceramide synthase (IPCS).

### 3 Very Long-Chain Fatty Acid Synthesis

The fatty acid component of plant ceramides consists of C16 fatty acids and very long-chain fatty acids (VLCFAs) with chain-lengths up to 26 carbon atoms. Interestingly, the fatty acid compositions of ceramide backbones of GlcCers and GIPCs have marked differences. In particular, GlcCers tend to have a higher content of C16 fatty acids compared to GIPCs, which are more enriched in VLCFAs (Sperling et al., 2005; Markham et al., 2006).

The VLCFA components of ceramides arise from acyl chain elongation in two carbon increments involving four reactions: (1) condensation of malonyl-CoA with an acyl-CoA chain, (2) reduction of the resulting 3-ketoacyl-CoA intermediate, (3) dehydration of the resulting 3-hydroxyacyl-CoA intermediate, and (4) reduction of the enoyl-CoA product of the dehydration reaction to form the two carbon elongated acyl-CoA chain (Blacklock and Jaworski, 2006).

Enzymes and corresponding genes for each of the four reactions have now been identified in plants (see Chapter 2). Genes for two classes of enzymes are capable of catalyzing the initial condensation reaction: 3-ketoacyl-CoA synthases (KCSs) and the structurally unrelated ELOs. The most characterized of these are the KCSs. Twenty-one KCS genes occur in the *Arabidopsis thaliana* genome, including *FAE1* (*FATTY ACID ELONGATION1*) or (*KCS19*) that is involved in the synthesis of VLCFAs found in seed oils and several *KCS*s that are associated with the synthesis of VLCFAs in surface waxes (e.g., *KCS1*, *KCS6* or *CER6*, and *KCS10* or *FDH*) (Joubes et al., 2008). A KCS that is specifically involved in sphingolipid VLCFA synthesis has yet to be identified. KCS-type enzymes do not occur in *Saccharomyces cerevisiae*. Instead, the initial condensation reaction for the synthesis of yeast VLCFAs, including those found in sphingolipids, involves ELO-type polypeptides (Toke and Martin, 1996). Four genes for ELO-related polypeptides occur in *Arabidopsis thaliana* (Table 1), but their in planta functions have yet to be reported. Although KCSs and ELOs share little sequence homology, plant KCSs can functionally replace ELOs in the elongation of fatty acids in *Saccharomyces cerevisiae*, suggesting that these enzymes have overlapping activities and can both interact with the other enzymes of the fatty acid elongation pathway for VLCFA synthesis (Paul et al., 2006).

Genes for the second reaction in fatty acid elongation, 3-ketoacyl-CoA reductase, have been identified as *GLOSSY8* alleles in maize (Dietrich et al., 2005), and an *Arabidopsis thaliana* homolog (At1g67730; Table 1) (Beaudoin et al., 2002) has been shown to complement the corresponding mutant (*ybr159*Δ) of *Saccharomyces cerevisiae*. Double mutants for the two maize *GLOSSY8* alleles develop kernels that contain normal endosperm, but the embryos of these seeds do not develop properly (Dietrich et al., 2005). In addition, kernels from the double mutant display large reductions in ceramide content (Dietrich et al., 2005).

Identification of genes for the 3-hydroxyacyl-CoA dehydratase, which catalyzes the dehydration step of fatty acid elongation, has long been elusive. However, a *Saccharomyces cerevisiae* enzyme Phs1p was recently shown to function as a dehydratase in fatty acid elongation (Denic and Weissman, 2007). The *Arabidopsis thaliana* homolog of the *PHS1* gene is the previously identified *PASTICCINO2* gene (At5g10480; Table 1) (Bach et al., 2008). Knockout mutants of this gene display embryo lethality, and the *pas2-1* partial mutant accumulates 3-hydroxyacyl-CoAs and has severe reductions in VLCFA content in sphingolipids, waxes, and seed oils (Bach et al., 2008).

The last step in fatty acid elongation, the reduction of the 2, 3-*trans* enoyl-CoA intermediate, is catalyzed by enoyl-CoA reductase. To date, only one enoyl-CoA reductase (At3g55360; Table 1) gene has been identified in *Arabidopsis thaliana* (Gable et al., 2004; Zheng et al., 2005), but it is probable that an additional enzyme(s) with enoyl-CoA reductase activity might be also present (Zheng et al., 2005). This possibility arises from the finding that knock-out of the enoyl-CoA reductase gene produces dwarfed mutants with an impairment in cell expansion that show a reduced content of VLCFAs in wax, seed oils and GlcCers, however, substantial amounts of VCLFAs are retained by the mutants (Zheng et al., 2005).

### 4 Ceramide Synthases

The final step in the assembly of ceramides is the condensation of a LCB with a fatty acid-CoA, a

reaction that is catalyzed by ceramide synthase (Fig. 3), sometimes referred to as sphinganine *N*-acyltransferase. Three genes for acyl-CoA-dependent ceramide synthases have been identified in *Arabidopsis thaliana* (At3g25540, *LOH1*; At3g19260, *LOH2*; and At1g13580, *LOH3*; Table 1) based on homology to yeast and mammalian ceramide synthases. The first ceramide synthase gene was identified in *Saccharomyces cerevisiae* as a locus whose deletion resulted in a 50% increase in cell life span (D'Mello et al., 1994). Based on this observation, the gene was designated *LONGEVITY ASSURANCE GENE1* or *LAG1*. *LAG1* and the related *LAC1* were subsequently shown to encode acyl-CoA-dependent ceramide synthases in *Saccharomyces cerevisiae* (Guillas et al., 2001; Schorling et al., 2001), and six homologs of *LAG1* and *LAC1* designated *LASS1-6* have been identified in human and mouse (Mizutani et al., 2005; Pewzner-Jung et al., 2006). Functional characterization of the *Arabidopsis thaliana* ceramide synthase-related genes has yet to be reported. Instead, the tomato *Asc-1* (*Alternaria stem canker-1*) gene that encodes a homolog of *LAG1* and *LAC1* has received attention because its presence results in resistance to the plant pathogen *Alternaria alternata* and to the mycotoxins fumonisin $B_1$ and *Alternaria alternata* f. sp. *lycopersici* (AAL) toxin (Brandwagt et al., 2000; Spassieva et al., 2002). It is now well established that these mycotoxins are potent inhibitors of acyl-CoA-dependent ceramide synthases, and that this inhibition results in accumulation of free LCBs (Abbas et al., 1994). As such, it appears that programmed cell death associated with *Alternaria alternata* infection of *asc-1* mutants is due to the buildup of cytotoxic free LCBs in response to the AAL toxin, and expression of the wild-type *Asc-1* gene is able to mitigate this effect (Brandwagt et al., 2002; Spassieva et al., 2002).

Recent analysis of intact sphingolipids from leaves of *Arabidopsis thaliana* points to patterns in the compositional makeup of ceramide backbones. For example, C16 fatty acids are more frequently paired with dihydroxy LCBs, and conversely, VLCFAs tend to be paired with trihydroxy LCBs (Markham and Jaworski, 2007; Chen et al., 2008). It is likely that these patterns reflect differing substrate specificities of the ceramide synthases found in plant cells. Although the substrate specificities of plant ceramide synthases have yet to be reported, the six mouse ceramide synthase genes, referred to as *Lass* (*Longevity assurance*) genes, have been shown to encode enzymes with distinct activities with fatty acyl-CoAs of differing chain lengths (Mizutani et al., 2005). For example, Lass6 is most active with C14 and C16 acyl-CoAs, while Lass2 displays the highest activity with C22 and C24 acyl-CoAs (Mizutani et al., 2005). Indirect evidence also points to the likelihood that ceramide synthases within a given plant species have different substrate specificities. In this regard, results from radiolabeling studies conducted with wild-type tomato and the *asc-1* mutant suggest that different molecular species of sphingolipids are produced in these lines (Spassieva et al., 2002). In addition, a recently described *Arabidopsis thaliana* mutant that produces only the dihydroxy form of LCBs accumulates primarily sphingolipids with ceramides that contain C16 fatty acids rather than VLCFAs (Chen et al., 2008). This observation is consistent with two functional classes of ceramide synthases in *Arabidopsis thaliana*: one class that primarily combines dihydroxy LCBs with C16 fatty acids and a second class that generates a preponderance of ceramides with trihydroxy LCBs and VLCFAs.

A second type of ceramide synthase activity was originally described in *Saccharomyces cerevisiae* that involves the condensation of a fatty acid and LCB through an acyl-CoA-independent mechanism (Mao et al., 2000a, b). This activity is catalyzed by the alkaline ceramidases YPC1 and YDC1. These enzymes function primarily in the breakdown of ceramides into free fatty acids and LCBs, but are also capable of catalyzing the reverse reaction to generate ceramides (Mao et al., 2000a, b). The so-called "reverse ceramidase" activity is not inhibited by fumonisin $B_1$, and expression of *YPC1* and *YDC1* rescues *Saccharomyces cerevisiae* cells from fumonisin $B_1$-induced growth inhibition (Mao et al., 2000a, b). Similar reverse ceramidase activity has been demonstrated in vitro with recombinant mammalian alkaline ceramidases (El Bawab et al., 2000, 2001). Although *Arabidopsis thaliana* contains a gene for a homolog of *Saccharomyces cerevisiae* alkaline ceramidases (At4g22330; Table 1), the quantitative significance, if any, of this enzyme for ceramide synthesis in vivo has yet to be determined.

## B Synthesis of Complex Sphingolipids

Most of the ceramides produced in plant cells subsequently serve as substrates for the attachment of polar head groups to form complex sphingolipids. These reactions consist predominately of the incorporation of a glucose residue to form GlcCer or the incorporation of an inositolphosphoryl moiety to form an inositolphosphoryl ceramide (IPC), the precursor of GIPCs.

### 1 Glucosylceramide Synthesis

The attachment of the glucose head group to a ceramide is catalyzed by GlcCer synthase (Fig. 3). Plant GlcCer synthase has received only limited study to date. A cotton GlcCer synthase cDNA was identified based on partial homology to the human GlcCer synthase and was shown to restore GlcCer production to a *Pichia pastoris* GlcCer synthase knockout mutant (Leipelt et al., 2001; Hillig et al., 2003). Interestingly, recombinant expression of the cotton GlcCer synthase not only generated GlcCers, but also produced small amounts of sterol glucosides in a *Pichia pastoris* GlcCer synthase/sterol glucosytransferase double mutant (Hillig et al., 2003). Whether this mixed activity occurs in planta remains to be determined. Based on enzyme assays conducted with the recombinant cotton enzyme, the glucose donor for the plant GlcCer synthase-mediated reaction is UDP-glucose (Hillig et al., 2003). Determination of whether GlcCers are essential in plants has been hindered by the lack of a T-DNA insertion mutant for the one putative GlcCer synthase gene (At2g19880; Table 1) in *Arabidopsis thaliana*.

### 2 Inositolphosphoceramide Synthesis

This synthesis of IPCs occurs via the transfer of the head group of phosphatidylinositol (PI) onto ceramide (Fig. 3). In addition to IPC, the second product of this reaction is diacylglycerol formed from the PI substrate. This activity is catalyzed by IPC synthase. Though IPC synthase activity has been assayed in plant extracts, the identification of plant genes for this enzyme has yet to be reported. In *Saccharomyces cerevisiae*, IPC synthase is encoded by the *AUR1* gene (Nagiec et al., 1997). This gene takes its name from the fact that *Saccharomyces cerevisiae* IPC synthase is strongly inhibited by aureobasidin A, as well as other fungal toxins including rustimicin and khafrefungin (Mandala et al., 1997, 1998b). IPC synthase activity in microsomes of wax bean (*Phaeolus vulgaris* L.) is also inhibited by aureobasidin A and rustimicin (Bromley et al., 2003). Despite this similarity in sensitivity to inhibitors, no homologs of the *Saccharomyces cerevisiae* IPC synthase have been identified to date in plants. Another possibility is that the plant gene is more closely related to the recently identified IPC synthases from protozoans. Indeed, homologs of these genes do occur in *Arabidopsis thaliana* (Table 1), and one of these genes has recently been shown to function as an IPC synthase (Wang et al., 2008). Following synthesis of IPCs, additional as yet uncharacterized glycosylation reactions presumably give rise to the more structurally complex GIPCs that are found in plants (Fig. 3).

## C Subcellular Location of Sphingolipid Synthesis

Our knowledge of the spatial layout of sphingolipid synthesis in plant cells is based on enzyme assays conducted with enriched membrane fractions and more recently on confocal microscopy of sphingolipid biosynthetic enzymes fused to fluorescent proteins. Studies with confocal microscopy have shown that the *Arabidopsis thaliana* LCB2 and LCB1 subunits of serine palmitoyltransferase reside in the ER (Tamura et al., 2001; Chen et al., 2006), as does the 3-ketosphinganine reductase (M. Chen and E.B. Cahoon, unpublished). These results, therefore, indicate that LCB synthesis occurs in the ER. Moreover, ceramide synthase activity has been identified in the ER in *Phaseolus vulgaris* seeds (Lynch and Dunn, 2004), which is consistent with the localization of the *Saccharomyces cerevisiae LAG1*- and *LAC1*-encoded ceramide synthases (Barz and Walter, 1999). In addition, glucosylceramide synthase activity in cotton was assigned to the ER (Hillig et al., 2003), while IPC synthase activity in *Phaseolus vulgaris* was detected in Golgi (Bromley et al., 2003), which is also the subcellular location of the *Saccharomyces cerevisiae* enzyme (Levine et al., 2000). Furthermore, a fluorescent protein tagged version of the recently identified IPC synthase from *Arabidopsis thaliana* was also localized in Golgi by confocal microscopy

(Wang et al., 2008). The picture that emerges is that ceramide synthesis occurs in the ER, but the sites of GlcCer and GIPC synthesis are physically separated between the ER and Golgi apparatus (Fig. 4).

The apparent spatial separation of GlcCer and GIPC synthesis may, in part, explain the structural differences found in the ceramide backbones of these complex sphingolipids. As noted earlier, the ceramides of GlcCers contain higher amounts of C16 fatty acids and dihydroxy LCBs. GIPCs, instead, are more enriched in ceramides with VLCFAs and trihydroxy LCBs. In the current model of sphingolipid synthesis in plants, two spatially separated and structurally divergent pools of ceramides would be required to support GlcCer and GIPC synthesis, and selective transport of specific ceramides to the Golgi for GIPC synthesis would be necessitated. This transport could be achieved by vesicular and/or non-vesicular mechanisms. Sphingomyelin synthesis in mammals is a relevant example of how non-vesicular transport of ceramides contributes to the biosynthetic processes of complex sphingolipids. In this example, ceramide is transported from its site of synthesis in the ER to the Golgi apparatus, where the phosphocholine head group of sphingomyelin is attached (Hanada et al., 2007). In mammals, ceramide trafficking from the ER to Golgi is mediated by ceramide transfer proteins (CERTs), which display specificity for different ceramide structures (Kudo et al., 2008). The demonstration of CERT-like proteins in plants has yet to be reported. However, the in vitro sphingolipid transfer properties of two polypeptides from *Arabidopsis thaliana* with homology to human and bovine glycolipid transfer proteins have been partially characterized. These polypeptides, designated ACD11 and AtGLTP1 (GLTP1, glycolipid transfer protein 1),

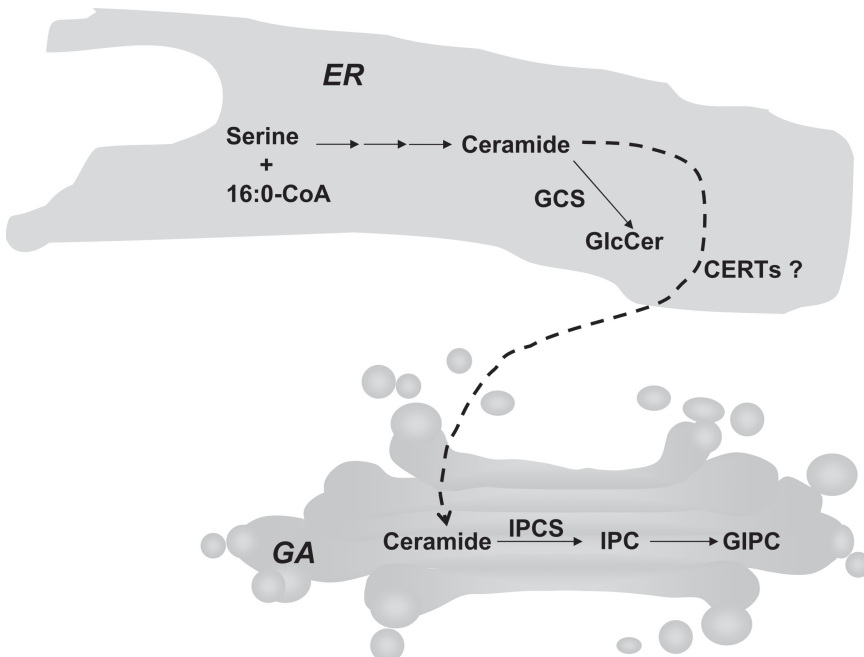


*Fig. 4.* Possible spatial separation of glucosylceramide (GlcCer) and glycosyl inositolphosphoceramide (GIPC) biosynthesis in plant cells. Evidence from assay of sphingolipid biosynthetic enzymes in enriched membrane fractions and from confocal microscopy of fluorescent protein-tags of these enzymes indicates that long-chain bases, ceramides, and GlcCers are synthesized primarily in the ER and inositolphosphoceramides (IPC) and likely GIPCs are synthesized in the Golgi apparatus (GA). Given that GlcCers and GIPCs have distinct ceramide composition, this model evokes the possibility that specific types of ceramides are transported by vesicular and/or non-vesicular routes (such as that mediated by CERTs in mammals) to the Golgi apparatus for GIPC synthesis. Abbreviations: GCS, glucosylceramide synthase; IPCS, inositolphosphoceramide synthase.

are encoded by At2g34690 and At2g33470, respectively. In the case of ACD11, the purified *E. coli*-expressed polypeptide was assayed for its ability to transfer [$^3$H] sphingolipids from a negatively charged donor phospholipid vesicles to a neutral acceptor vesicles. Of the three sphingolipids tested, the highest activity was measured with the sphingosine (d18:1Δ4). The protein displayed > ten-fold lower activity with galactosylceramide and ceramide. It should be noted that sphingosine is typically of very low abundance relative to other LCBs in *Arabidopsis thaliana*, and galactosylceramides are not known to occur in *Arabidopsis thaliana*. For AtGLTP1, the purified recombinant protein was assayed for its ability to transfer fluorescent sphingolipids between phospholipid or galactolipid vesicles (West et al., 2008). These studies revealed that AtGLTP1 is about eight times more active with glucosylceramide than with galactosylceramide and about 30 times more active with glucosylceramide than with lactosylceramide (West et al., 2008). These findings provide compelling evidence for a role of ACD11 and AtGLTP1 in sphingolipid transfer in *Arabidopsis thaliana* cells. Still, the in vivo substrates and the in planta contributions of these proteins to the transport of sphingolipids among different membranes (e.g., ER to Golgi) remain to be determined.

## D Long-Chain Base Modification Reactions

The eight LCBs that derive from sphinganine (d18:0) are formed by at least one of three enzymes: (1) LCB C-4 hydroxylase, (2) LCB Δ8 desaturase, and (3) LCB Δ4 desaturase (Fig. 5). These enzymes are non-heme diiron oxo proteins that contain the three histidine "boxes" that are characteristic of fatty acid desaturases and hydroxylases, and other members of this enzyme family (Shanklin and Cahoon, 1998). The LCB Δ8 desaturase is particularly intriguing because it typically can introduce the Δ8 double bond of LCBs in either the *cis* or *trans* orientations (Sperling et al., 1998; Beckmann et al., 2002). By contrast, the LCB Δ4 desaturase lacks this bifunctional activity and instead introduces a double bond at the Δ4 position exclusively in the *trans* orientation (Ternes et al., 2002). Although each of these enzymes can function alone to generate either saturated trihydroxy LCBs (i.e., t18:0) or monounsaturated dihydroxy LCBs (i.e., d18:1Δ4*trans*, d18:1Δ8*trans*, and d18:1Δ8*cis*), four of the nine LCBs found in plants are the products of the combined activities of two of these enzymes. In this regard, the activities of the LCB C-4 hydroxylase and the LCB Δ8 desaturase give rise to the two most abundant LCBs in *Arabidopsis thaliana* leaves: t18:1Δ8*trans* and t18:1Δ8*cis*. In addition, the concerted activities of the LCB Δ4 desaturase and the LCB Δ8 desaturase produce the diunsaturated dihydroxy LCBs d18:2Δ4*trans*,8*trans* and d18:2Δ4*trans*,8*cis*, which are typically enriched in GlcCers of plants, such as tomato. To date, the plant LCB C-4 hydroxylase and LCB Δ8 desaturases have been the most extensively characterized.

### 1 Long-Chain Base C-4 Hydroxylation

The LCB C-4 hydroxylase catalyzes the introduction of a hydroxyl group at the C-4 position of a dihydroxy LCB to form a trihydroxy LCB (Fig. 5a). Homologs of the *Saccharomyces cerevisiae* LCB C-4 hydroxylase gene *SUR2* occur in plants (Sperling et al., 2001; Imamura et al., 2007; Chen et al., 2008). Two LCB C-4 hydroxylase genes have been identified in *Arabidopsis thaliana* (At1g69640, *SBH1*; At1g14290, *SBH2*; Table1) and both have been shown to restore the synthesis of trihydroxy LCBs when expressed in *Saccharomyces cerevisiae sur2* mutants (Sperling et al., 2001). Although definitive evidence has yet to be reported regarding the nature of the substrate for plant LCB C-4 hydroxylases, it is likely that C-4 hydroxylation occurs primarily on free dihydroxy LCBs prior to incorporation into ceramide (Wright et al., 2003) and the trihydroxy LCB t18:0 is the predominate free LCB in *Arabidopsis thaliana* leaves (Markham and Jaworski, 2007; Chen et al., 2008). The functional significance of LCB C-4 hydroxylation in *Arabidopsis thaliana* was recently examined by the generation of double mutants and RNAi suppression lines for the two hydroxylase genes (Chen et al., 2008). Based on the lack of a growth phenotype in the *Saccharomyces cerevisiae sur2* mutant (Haak et al., 1997; Grilley et al., 1998), it was anticipated that the *Arabidopsis thaliana* mutants would have no obvious phenotypes. Instead, double mutants were found to be severely dwarfed and did not progress from vegetative to reproductive growth

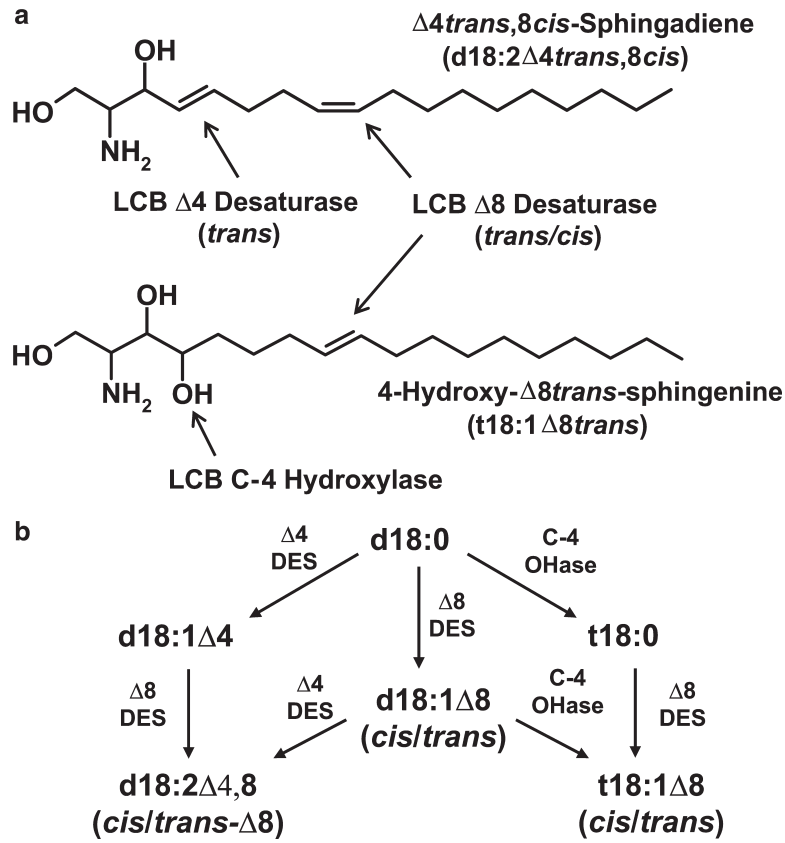


*Fig. 5.* Long-chain base modification reactions in plants. **(a)** The C-4 hydroxyl group and Δ4 and Δ8 double bonds that are found in plant long-chain bases (LCBs) arise from the concerted activities of the LCB C-4 hydroxylase and LCB Δ4 and Δ8 desaturases, respectively. **(b)** These enzymes can work separately or in combination to generate the complete complement of LCBs found in plant sphingolipids. The LCB Δ8 desaturase can function to introduce a double bond in either the *cis* or *trans* configuration, and the LCB Δ4 desaturase functions exclusively as a *trans* desaturase. Of note, the sequence of these reactions and the exact nature of the substrates (e.g., free LCB, ceramide) have yet to be definitively established. C-4 OHase, LCB C-4 hydroxylase. Δ4 DES, LCB Δ4 desaturase. Δ8 DES, LCB Δ8 desaturase.

(Chen et al., 2008). In addition, the degree of growth reduction in RNAi lines was found to be more severe as the relative content of trihydroxy LCBs decreased. Unexpectedly, the sphingolipid content in double mutants was 2.5- to three-fold higher than in wild type plants, and the accumulation of sphingolipids was primarily the result of increased amounts of molecular species with C16 fatty acids, rather than the more typical VLCFAs, in all sphingolipid classes (Chen et al., 2008). As described above, the increased levels of ceramide backbones with C16 fatty acids and dihydroxy LCBs is likely reflective of the substrate specificities of ceramide synthases in *Arabidopsis thaliana*. Overall, these results indicate that LCB C-4 hydroxylation is a critical sphingolipid structural modification for growth and for the regulation of sphingolipid content and composition in *Arabidopsis thaliana*. These findings also suggest that the synthesis of trihydroxy LCBs is important for mediating flux into the sphingolipid biosynthetic pathway, perhaps through regulation of SPT activity, to meet the demands for growth.

### *2 Long-Chain Base Δ8 Desaturation*

The LCB Δ8 desaturase is absent in *Saccharomyces cerevisiae* and mammalian cells, but does occur in plants and many fungi. The LCB Δ8 desaturase was first identified in plants as a novel peptide consisting of an *N*-terminal cytochrome $b_5$ domain fused to a desaturase-like polypeptide

(Sperling et al., 1998). Heterologous expression of the *Arabidopsis thaliana* and *Brassica napus* cDNAs in *Saccharomyces cerevisiae* allows the biosynthesis of *cis* and *trans* isomers of t18:1Δ8 (Sperling et al., 1998). These results demonstrate that the plant LCB Δ8 desaturase is bifunctional with regard to the stereospecificity of double bond insertion. In most plants, the *trans* isomers of Δ8 unsaturated LCBs predominate in the total sphingolipid extract. However, the *cis* isomer of t18:1Δ8 is much more enriched in GlcCers relative to GIPCs in plants, such as *Arabidopsis thaliana* (Sperling et al., 2005; Markham et al., 2006; Markham and Jaworski, 2007). It is not yet clear if this difference in stereoisomer composition results from distinct LCB Δ8 desaturases that may be associated with GlcCers and GIPCs. Recently, a plant LCB Δ8 desaturase from the legume *Stylosanthes hamata* was shown to produce more of the *cis* isomer of t18:1Δ8 when expressed in *Saccharomyces cerevisiae* (Ryan et al., 2007). This is in contrast to findings with the *Arabidopsis thaliana* and *Brassica napus* enzymes, which generated mostly the *trans* isomer upon expression in *Saccharomyces cerevisiae* (Sperling et al., 1998). These studies demonstrate that LCB Δ8 desaturase with different stereoselective properties have evolved in plants. Mechanistic studies with the sunflower LCB Δ8 desaturase indicate that the bifunctionality of this enzyme arises from two different conformations that the LCB substrate can assume in the active site (Beckmann et al., 2002). Interestingly, expression of the *Stylosanthes hamata* desaturase in *Arabidopsis thaliana* was shown to not only increase the content of t18:1Δ8cis but also confer increased tolerance to aluminum toxicity (Ryan et al., 2007). This finding strongly indicates that the relative amounts of *cis–trans* isomers of unsaturated LCBs in sphingolipids impact the plant's ability to adapt to at least some abiotic stresses. However, it has yet to be established if LCB Δ8 unsaturation is essential in plants. A number of metabolic questions regarding the LCB Δ8 desaturase also remain unanswered. For example, it is not known if this enzyme uses a free LCB, ceramide, or complex sphingolipid as a substrate. In addition, it has not been established if distinct LCB Δ8 desaturases are involved in the synthesis of monounsaturated and diunsaturated LCBs. This is particularly relevant given the occurrence of two LCB Δ8 desaturase genes in *Arabidopsis thaliana* (At3g61580, *SLD1*; At2g46210, *SLD2*; Table 1).

### *3 Long-Chain Base Δ4 Desaturation*

The LCB Δ4 desaturase introduces the *trans*-Δ4 unsaturation that is found in GlcCers of many plant species, but is typically absent in GIPCs (Fig. 5) (Markham et al., 2006). In contrast to mammals, Δ4 monounsaturated LCBs are present in low abundance in plant sphingolipids (Sperling et al., 2005; Markham et al., 2006). Instead, Δ4 double bonds occur together with *cis*- or *trans*- Δ8 double bonds in the diunsaturated LCB d18:2Δ4,8 (sphingadiene). The plant LCB Δ4 desaturase is most related to animal LCB Δ4 desaturases, including the *Drosophila melanogaster* Δ4 desaturase encoded by the *DEGENERATIVE SPERMATOCYTE-1* gene (*DES-1*) (Ternes et al., 2002). In addition, the mouse LCB Δ4 desaturase DES2 has been shown to act as a bifunctional enzyme that can also catalyze C-4 hydroxylation of dihydroxy LCBs (Omae et al., 2004). This gene family also includes LCB Δ4 desaturases from a number of fungal species (Ternes et al., 2002). Functional identification of plant LCB Δ4 desaturases has only recently been reported. In this study, the *Arabidopsis thaliana* LCB Δ4 desaturase (At4g04930, Table 1) was shown to restore the synthesis of d18:2 to a *Pichia pastoris* Δ4 desaturase null mutant (Michaelson et al., 2009). It is notable that functional expression of plant LCB Δ4 desaturases has not been achievable in *Saccharomyces cerevisiae* (Michaelson et al., 2009). This may be reflective of the substrate specificity of the plant Δ4 LCB desaturase. Because Δ4 unsaturation is found almost entirely in GlcCers, one possibility is that the LCB Δ4 desaturase uses GlcCers as substrates. However, GlcCers do not occur in *Saccharomyces cerevisiae*. Another possibility is that the Δ4 desaturase activity requires the presence of a Δ8 double bond in LCB substrates, given that Δ4 monounsaturated LCBs are of low abundance in plants. Like the previous metabolic scenario, *Saccharomyces cerevisiae* does not have a Δ8 desaturase, and to our knowledge, the co-expression of plant LCB Δ4 and Δ8 desaturases in *Saccharomyces cerevisiae* has not been reported.

As with the LCB C-4 hydroxylase and Δ8 desaturase, the substrate for the plant LCB Δ4

desaturase has yet to be defined. In addition to the possibility that this enzyme uses a GlcCer substrate (see above), it cannot be ruled out that the Δ4 unsaturation is introduced into ceramides, which are then selectively used for GlcCer synthesis. The latter hypothesis is supported by the observation that in *Pichia pastoris* knock-out of the LCB Δ4 desaturase results in a complete loss of GlcCers (Michaelson et al., 2009). This observation supports the idea that Δ4 unsaturation is introduced prior to the attachment of the glucose head group and as such, is necessary for channeling of ceramides into GlcCers in fungi.

The functional significance of the LCB Δ4 desaturase in plants is also not clear. Some plants are enriched in Δ4 unsaturated LCBs in GlcCers (e.g., tomato), whereas other species (e.g., *Arabidopsis thaliana*) contain very low levels of these LCBs in GlcCers (Markham et al., 2006). The near absence of Δ4 unsaturated sphingolipids in *Arabidopsis thaliana* suggests that the LCB Δ4 desaturase may be of little importance in this plant. Indeed, a T-DNA knock-out of the corresponding gene (At4g04930) did not affect the growth and development of *Arabidopsis thaliana* (Michaelson et al., 2009). In addition, changes in stomatal aperture in response to ABA treatment was also not affected in the *Arabidopsis thaliana* LCB Δ4 knockout mutant relative to the wild-type control (Michaelson et al., 2009). This finding brings into question the purported role of Δ4 unsaturated LCB-1-phosphates in ABA-dependent stomatal closure. Still, it cannot be ruled out that the Δ4 desaturase has some important physiological functions in plants, such as tomato, that contain high levels of these LCBs in GlcCers.

### *E Fatty Acid α-Hydroxylation*

A distinctive feature of the ceramide component of sphingolipids of most eukaryotes, including plants, is the presence of a α-hydroxyl group on the fatty acid moiety. In plant species characterized to date, most of the fatty acids of sphingolipids contain this structural feature, which results from the activity of the sphingolipid fatty acid α-hydroxylase. The α-hydroxylase gene (*FAH1* or *SCS7*) was first discovered in *Saccharomyces cerevisiae* (Haak et al., 1997; Mitchell and Martin, 1997). By homology, one of the two *Arabidopsis thaliana* α-hydroxylase genes (At2g34770; Table 1) was identified and subsequently shown to restore α-hydroxylation of 26:0 upon expression in the corresponding *Saccharomyces cerevisiae* mutant (Mitchell and Martin, 1997). Like the LCB Δ8 desaturase, the *Saccharomyces cerevisiae* α-hydroxylase contains a cytochrome $b_5$ domain at its *N*-terminus (Mitchell and Martin, 1997). This domain, however, is absent in the plant sphingolipid fatty acid α-hydroxylases identified to date (Mitchell and Martin, 1997). As with the LCB modification reactions described above, the substrate for fatty acid α-hydroxylation has not been established in plants, nor has it been determined in yeast or mammals. Results from studies conducted in *Tetrahymena pyriformis* point to α-hydroxylation occurring on the intact complex sphingolipid and/or ceramides rather than on free fatty acids or acyl-CoAs prior to incorporation into ceramides (Kaya et al., 1984). Notably, pools of non-hydroxy ceramides and hydroxy ceramides are detectable in *Arabidopsis thaliana* leaves (Markham and Jaworski, 2007; Chen et al., 2008). In contrast to the high content of α-hydroxy fatty acids in *Arabidopsis thaliana* complex sphingolipids (i.e., GlcCers and GIPCs), ceramides lacking hydroxylated fatty acids are approximately three times more abundant than ceramides with hydroxylated fatty acids (Markham and Jaworski, 2007; Chen et al., 2008). This observation is consistent with α-hydroxylation occurring after incorporation of the fatty acid into ceramides in plants. The functional significance of sphingolipid fatty acid α-hydroxylation is not known. *Saccharomyces cerevisiae* mutants devoid of sphingolipid α-hydroxy fatty acids are viable and lack noticeable growth defects (Haak et al., 1997; Mitchell and Martin, 1997). As with the LCB Δ8 and Δ4 desaturases, determination of the function of sphingolipid α-hydroxylation in plants awaits the identification of null mutants for this structural modification.

### *F Long-Chain Base-1-Phosphates: Synthesis and Turnover*

Long-chain base-1-phosphates (LCB-Ps; Fig. 1) are bioactive metabolites that are generated from the phosphorylation of LCBs arising either from de novo synthesis or from the catabolism of sphingolipids (Fig. 3). LCB-Ps in mammals and *Saccharomyces cerevisiae* have been shown to

function as regulators of diverse activities including cell proliferation, differentiation, and apoptosis (Zhang et al., 1991; Mao et al., 1999; Mandala et al., 2000; Le Stunff et al., 2002a; Johnson et al., 2003; Taha et al., 2006). Plant LCB-Ps have received attention because of evidence linking these molecules to signal transduction in ABA-dependent stomatal closure (Ng et al., 2001; Coursol et al., 2003; Worrall et al., 2003). The levels of LCB-Ps in plant cells are controlled primarily by the biosynthetic activity of LCB kinases and the catabolic activities of LCB-P phosphatases and lyases. Based on the reported role of LCB-Ps in guard cell function (Ng et al., 2001; Coursol et al., 2003; Worrall et al., 2003) and plant cell death (Shi et al., 2007), the interplay of these activities may be important for triggering or attenuating key physiological processes in plants.

### *1 Long-Chain Base Phosphorylation*

LCB-Ps are synthesized by phosphorylation of the C-1 position of free LCBs through the activity of LCB kinases (Fig. 3). In *Saccharomyces cerevisiae*, two LCB kinases, LCB4 and LCB5, have been identified. Of these, LCB4 accounts for approximately 97% of the LCB kinase activity in *Saccharomyces cerevisiae* cells (Nagiec et al., 1998). Similarly, two sphingosine kinases (SphK1 and SphK2) occur in mammals (Liu et al., 2000, 2002). *Arabidopsis thaliana* contains four genes with homology to the mammalian *SphK1*: At5g23450 (*AtLCBK1*), At4g21540 (*SPHK1*), At2g46090 (*SPHK2*), and At5g51290 (*ACD5* or *AtCERK*) (Table 1). Of these genes, *AtLCBK1* was the first to be shown to encode a functional LCB kinase (Imai and Nishiura, 2005). The *Escherichia coli*-expressed AtLCBK1 phosphorylated a range of naturally occurring LCBs, including d18:0, d18:1Δ4, d18:2Δ4,8, and t18:0 (Imai and Nishiura, 2005). At4g21540 was also recently shown to encode a functional LCB kinase (Worrall et al., 2008). Triton X-100-solubilized extracts from human embryonic kidney 293 cells expressing this gene were able to phosphorylate d18:0, t18:0, t18:1Δ8, and d18:2Δ4,8 (Worrall et al., 2008). This gene was designated *SPHK1* because of its in vitro activity with sphingosine (or Δ4*trans*-sphingenine, d18:Δ4*trans*) and its likely relation to previous studies of the role of sphingosine-1-P in mediation of stomatal closure (Worrall et al., 2008). Despite this, sphingosine is a very minor LCB in most organs of *Arabidopsis thaliana* and is likely not the primary in vivo substrate of this enzyme. In contrast to the findings with SPHK1, recombinant expression of At2g46090, designated *SPHK2*, in human embryonic kidney 293 cells failed to yield an active protein (Worrall et al., 2008). As such, it is unclear if SPHK2 is a true LCB kinase in *Arabidopsis thaliana*. The final LCB kinase-like gene in *Arabidopsis thaliana* At5g51290 was demonstrated to encode a ceramide kinase rather than an LCB kinase (see below) (Liang et al., 2003).

The two demonstrated LCB kinases in *Arabidopsis thaliana* AtLCBK1 and SPHK1 contain a putative ATP-binding domain (GDGXXXEXXNGXXXR), which is also conserved in other SPHK kinases from fungi and animals (Liu et al., 2002). Interestingly, the in vitro substrate profiles of AtLCBK1 and SPHK1 do not perfectly overlap (Imai and Nishiura, 2005; Worrall et al., 2008). Of most significance, the activity of recombinant AtLCBK1 with t18:0 is about 20% of that with d18:1Δ4 (Imai and Nishiura, 2005). By comparison, SPHK1 is nearly equally active with d18:0 and t18:0 (Worrall et al., 2008). In addition, a T-DNA knockout of *SPHK1* resulted in the loss of nearly all sphingosine phosphorylation activity in membrane extracts from *Arabidopsis thaliana* leaves (Worrall et al., 2008). This suggests that SPHK1, rather than AtLCBK1, contributes primarily to the LCB kinase activity in *Arabidopsis thaliana* leaves, although AtLCBK1 was shown to be constitutively expressed (Imai and Nishiura, 2005).

The subcellular localization of the plant LCB kinase has not been fully established. In *Saccharomyces cerevisiae*, there are conflicting reports regarding the localization of these enzymes. For example, LCB4 was localized to ER, Golgi, and late endosomes using an epitope-tagging strategy (Hait et al., 2002; Funato et al., 2003), but this enzyme was found on the cell perimeter using immunofluorescence microscopy with antibodies against LCB4 (Kihara et al., 2005). LCB kinase activity in plants is primarily membrane associated, and this activity has been identified in ER and Golgi-enriched fractions from maize microsomes (Crowther and Lynch, 1997; Coursol et al., 2005; Worrall et al., 2008). Specific subcellular

localization of AtLCBK1 and SPHK1 polypeptides awaits further study.

The in planta functions of LCB kinases have been explored by the characterization of *Arabidopsis thaliana SPHK1* overexpression lines and lines containing a T-DNA disruption in *SPHK1*. In these studies, stomatal aperture was greater in the T-DNA disruption line and smaller in the overexpression line relative to a wild-type control in response to exogenous ABA treatment (Worrall et al., 2008). These results are consistent with a role of LCB-Ps in ABA-dependent stomatal closure. In addition, seeds from the *SPHK1*-overexpression line displayed slower germination than seeds from wild-type plants (Worrall et al., 2008). Conversely, seeds from the *SPHK1*-disruption line displayed faster germination than the control. The delayed germination of seeds from the overexpression line was enhanced by exogenous ABA (Worrall et al., 2008). These results point to a role of LCB-Ps in mediating germination, and this activity may be a component of ABA signaling processes.

### *2 Long-Chain Base-1-Phosphate Catabolism*

The turnover of LCB-Ps can proceed either by dephosphorylation to form free LCBs via the activity of LCB-P phosphatases (Mao et al., 1997) or by hydrolysis of the LCBs to generate long-chain aldehydes and phosphoethanolamine via the activity of LCB-P lyases (Saba et al., 1997) (Fig. 3). Two LCB-P phosphatase genes *YSR2* (or *LCB3*) and *YSR3* and one LCB-P lyase gene *DPL1* occur in *Saccharomyces cerevisiae* (Qie et al., 1997; Mandala et al., 1998a; Mao et al., 1999; Bach et al., 2008). Deletion of either of the two LCB-P phosphatase genes results in the accumulation of primarily d18:0-1-P, but does not result in any detectable alterations in growth (Mandala et al., 1998a; Mao et al., 1999). Conversely, overexpression of *YSR2* suppresses growth of *Saccharomyces cerevisiae* cells (Mao et al., 1999). A notable phenotype associated with disruption of *YSR2* is enhanced thermotolerance, which suggests a role of LCB-Ps in stress adaptation in *Saccharomyces cerevisiae* (Mao et al., 1999). The *YSR2* mutant is also incapable of synthesizing sphingolipids from exogenous LCBs (Qie et al., 1997). The *Saccharomyces cerevisiae* LCB-P lyase gene *DPL1* (or *BST1*) was initially identified as a gene whose overexpression could rescue cells from growth inhibition by exogenous sphingosine (Saba et al., 1997). As observed with *YSR2* and *YSR3*, disruption of *DPL1* results in the accumulation of LCB-Ps (Saba et al., 1997). Interestingly, double mutants of *DPL1* and *YSR2* are non-viable, but can be rescued by knockout of the LCB kinase gene *LCB4* (Kim et al., 2000). These observations indicate that high levels LCB-Ps can result in lethality in *Saccharomyces cerevisiae*.

Based on homology to the *Saccharomyces cerevisiae YSR2*, *YSR3*, and *DPL1* genes, *Arabidopsis thaliana* contains at least one LCB-P phosphatase (*AtLCB-PP1*, At3g58490; Table 1) gene and one LCB-P lyase gene (*AtDPL1*, At1g27980; Table 1). Although the *AtLCB-PP1*-encoded polypeptide shares only moderate homology with *YSR2* and *YSR3*, it contains a phosphohydrolase domain that is found in LCB-P phosphatases from other eukaryotes (Le Stunff et al., 2002b). Biochemical characterization of the polypeptide encoded *AtLCB-PP1* or homologs from other plant species has yet to be reported. However, the *Arabidopsis thaliana DPL1* homolog *AtDPL1* has been studied in detail (Niu et al., 2007; Tsegaye et al., 2007). Expression of the *AtDPL1* gene is able to rescue the growth inhibition of the *Saccharomyces cerevisiae dpl1* mutant in response to exogenous LCBs (Niu et al., 2007; Tsegaye et al., 2007). The AtDPL1 polypeptide also restores LCB-P lyase activity to the *Saccharomyces cerevisiae dpl1* mutant (Tsegaye et al., 2007). This enzyme, like LCB kinases, has membrane-associated activity, and a fluorescent protein fusion of this polypeptide localizes to the ER (Tsegaye et al., 2007). In addition, T-DNA mutants for the *AtDPL1* gene lack LCB-P lyase activity and show increased accumulation of LCB-Ps (Tsegaye et al., 2007). These plants do not display growth phenotypes but are hypersensitive to fumonisin $B_1$, which is likely due to higher levels of LCBs and LCB-Ps accumulation in the mutant in response to the mycotoxin relative to wild-type plants (Tsegaye et al., 2007). It is also notable that expression of *AtDPL1* is strongly upregulated in response to senescence (Niu et al., 2007; Tsegaye et al., 2007). This implies that sphingolipid turnover is an active process during senescence or alternatively that modulation of LCB-P levels plays some physiological role during senescence. Although *AtDPL1* mutants have now been characterized,

the overall importance of LCB-P turnover in *Arabidopsis thaliana* awaits the generation of LCB-P phosphatase and lyase mutants that completely lack the ability to degrade LCB-Ps.

### G Ceramide Phosphorylation

Phosphorylation of ceramides has become a topic of interest because of the link between this reaction and programmed cell death observed in the *acd5* mutant of *Arabidopsis thaliana*. As described in more detail below, mutants of the *ACD5* gene (*AtCERK*, At5g51290; Table 1) display early senescence and a spontaneous increase in salicylic acid and enhanced expression of pathogenesis-related genes, presumably due to the buildup of free ceramides. The *ACD5* gene encodes a polypeptide that is most closely related to mammalian ceramide kinases and more distantly related to LCB kinases (Liang et al., 2003). Consistent with this identification, the *ACD5*-encoded enzyme displays ceramide kinase activity that introduces a phosphate group at the C-1 position of a free ceramide to form ceramide-1-phosphate (Liang et al., 2003) (Fig. 3). Partially purified enzyme from expression of *ACD5* in *Escherichia coli* was most active with ceramides containing C6 or C8 fatty acids coupled to a dihydroxy LCB (Liang et al., 2003). The enzyme showed little or no activity with sphingosine or diacylglycerol, indicating that it is neither an LCB kinase nor a diacylglycerol kinase (Liang et al., 2003). The most obvious function of the ceramide kinase is to mediate programmed cell death by regulating the relative amounts of free and phosphorylated ceramides. No other function has yet been ascribed to the plant ceramide kinase, and it is not known if a ceramide phosphatase functions in combination with the plant ceramide kinase, in a manner analogous to LCB kinases and phosphatases.

## IV Sphingolipid Turnover

### A Ceramide Turnover

Ceramidases catalyze the catabolism of ceramides by cleaving amide bonds to yield LCBs and fatty acids (Fig. 3). Three classes of ceramidases have been reported in eukaryotes whose activities differ based on pH. These enzymes are classified as acid, neutral and alkaline ceramidases. The human acid ceramidase is the first purified and characterized enzyme of the acid ceramidase class (Sugita et al., 1972). Mutations in the corresponding gene are the basis for a lysosomal disorder known as Farber disease (Koch et al., 1996; Ferlinz et al., 2001). There is no apparent homolog of human acid ceramidase in *Arabidopsis thaliana* based on homology searches using the human acid ceramidase as query.

Neutral ceramidases catalyze the hydrolysis of ceramide in the neutral pH range; however, they also have the abilities to catalyze the reverse reaction of ceramide synthesis by using a LCB and free palmitic acid as substrates. As described above, this activity is not inhibited by fumonisin $B_1$, in contrast to the acyl-CoA-dependent ceramide synthase (El Bawab et al., 2001). From database searches for homologs of the human neutral ceramidase *ASAH2*, three candidate neutral ceramidase genes occur in *Arabidopsis thaliana* (At1g07380, At2g38010, and At5g58980; Table 1), and only one candidate neutral ceramidase occurs in rice (*OsCDase*, Os01g0624000). All of the putative *Arabidopsis thaliana* and rice neutral ceramidase polypeptides contain the conserved peptide sequence GDVSPN in their amidase catalytic domain NXGDVSPNXXC (Galadari et al., 2006; Pata et al., 2008). To date, the rice *OsCDase* gene is the only plant neutral ceramidase-like gene that has been characterized. The recombinant OsCDase enzyme generated in *Saccharomyces cerevisiae* ceramidase mutants was more active with a fluorescent-labeled C12-d18:1Δ4 substrate than with a C12-t18:0 substrate and was active over a broad pH range, with maximal activity at pH 5.7 to 6.0. In addition, expression of the rice enzyme in *Saccharomyces cerevisiae* was accompanied by an increase in ceramides containing very-long chain fatty acids with t18:0 LCBs. The increase in these ceramides, rather than a decrease that would be expected with ceramidase activity, suggests that OsCDase may also have ceramide synthase (or reverse ceramidase) activity. In addition, the fluorescent protein-tagged OsCDase was shown to localize in ER and Golgi apparatus (Pata et al., 2008). This finding is somewhat unexpected because the bulk of sphingolipids in plant cells is believed to be associated with the plasma

membrane and tonoplast. As such, it is unclear at this point if neutral ceramidases are involved in sphingolipid turnover in planta or have other functions in plant cells.

The final type of eukaryotic ceramidase is the alkaline ceramidase. In *Saccharomyces cerevisiae*, two alkaline ceramidase genes *YPC* and *YDC* have been identified (Mao et al., 2000a, b). *YPC* was isolated from a screen designed to identify genes that would confer fumonisin $B_1$ resistance to *Saccharomyces cerevisiae*. The YPC polypeptide was subsequently shown to function as both an alkaline ceramidase and as an acyl-CoA-independent ceramide synthase (Mao et al., 2000a). YPC and YDC have distinct in vitro substrate preferences. YPC is more active with ceramide substrates containing t18:0 (Mao et al., 2000a), whereas YDC is more active with ceramide substrates d18:0 (Mao et al., 2000b). *Arabidopsis thaliana* contains one uncharacterized homolog of *YPC* and *YDC* (At4g22330; Table 1).

### *B Complex Sphingolipid Turnover*

GlcCers and GIPCs are the two most abundant sphingolipid classes in plants, yet little is known about plant catabolic enzymes associated with the removal of their head groups. These activities are likely to be critical for the maintenance of the optimal content and composition of sphingolipids in membranes. The degradation of GlcCers in mammals is catalyzed by glucosylceramidase (or cerebrosidase), which converts glucosylceramide to ceramide and glucose by cleavage of the glycosidic linkage between these moieties (Brady et al., 1965) (Fig. 3). Two forms of glucosylceramidase occur in mammals: lysosomal glucosylceramidase and non-lysosomal glucosylceramidase. Mutation in the human lysosomal glucosylceramidase is the genetic basis for Gaucher's disease, which is characterized by hyperaccumulation of GlcCers, particularly in spleen (Ho and O'Brien, 1971). No homolog of the human lysosomal glucosylceramidase is detectable in the *Arabidopsis thaliana* genome database. A non-lysosomal glucosylceramidase was recently identified as the earlier described bile acid β-glucosidase (GBA2) in mice (Yildiz et al., 2006; Boot et al., 2007). At least four homologs of GBA2 occur in *Arabidopsis thaliana* (Table 1) that share 40–50% amino acid sequence similarity with the mammalian GBA2. The activities of the corresponding *Arabidopsis thaliana* enzymes have not been reported.

Turnover of GIPCs, the most abundant sphingolipid class in *Arabidopsis thaliana*, has not been determined (Fig. 3). In the *Saccharomyces cerevisiae*, removal of the phosphoinositol head group of inositol phosphate-containing sphingolipids is catalyzed by the ISC1 polypeptide that was identified based on limited homology with bacterial neutral sphingomyelinases (Sawai et al., 2000; Matmati and Hannun, 2008). The bacterial enzymes catalyze the release of the phosphocholine head group of sphingomyelin to generate free ceramide. However, no homolog of the *Saccharomyces cerevisiae ISC* is detectable in database searches of *Arabidopsis thaliana*. In mammals, acid, alkaline, and neutral classes of sphingomyelinases have been identified (Marchesini and Hannun, 2004; Duan, 2006; Ohlsson et al., 2007). While *Arabidopsis thaliana* lacks homologs of mammalian acid and neutral sphingomyelinases, three tandemly arranged genes that encode as yet uncharacterized polypeptides with 30% identity to the human intestinal alkaline sphingomyelinase occur in *Arabidopsis thaliana* (At4g29680, At4g29690, At4g29700; Table 1).

Overall, catabolic reactions are undoubtedly of importance for maintenance of sphingolipid homeostasis and for the generation of bioactive metabolites in plant cells. However, the turnover of sphingolipids in plants has received only limited characterization to date. As such, catabolic enzymes, including those involved in ceramide degradation and removal of head groups of complex sphingolipids will likely be key targets for future research of sphingolipid metabolism in plants.

## V Sphingolipid Function

### *A Sphingolipids as Membrane Structural Components*

#### *1 Distribution and Functions of Sphingolipids in Membranes*

Based on research conducted in the 1950s and early 1960s (Carter et al., 1958a, b, 1960, 1961), it is now recognized that GIPCs and GlcCers are

the principal complex sphingolipids in plants (Fig. 1). More recently, GIPCs and GlcCers were determined to compose approximately 60% and 30% of the sphingolipids of *Arabidopsis thaliana* leaves, respectively (Markham et al., 2006). The remainder of the sphingolipids in *Arabidopsis thaliana* leaves consists of ceramides, free LCBs, and LCB-1-Ps. Measurement of the complete complement of sphingolipids in specific membranes in plants, however, has yet to be reported. Through the use of indirect measurements, sphingolipids have been estimated to compose >40% of the total lipids in plasma membrane in various plant species (Sperling et al., 2005). In addition, numerous reports have identified GlcCers as major lipid constituents of plasma membrane, tonoplast, Golgi apparatus, and detergent resistant membrane fractions isolated from plasma membrane and Golgi (e.g., Verhoek et al., 1983; Yoshida and Uemura, 1986; Lynch and Steponkus, 1987; Haschke et al., 1990; Cahoon and Lynch, 1991; Mongrand et al., 2004; Sperling et al., 2005; Laloi et al., 2007). For example, GlcCers reportedly compose 30% of the total lipids in plasma membrane from spring oat (Uemura and Steponkus, 1994) and nearly 20% of the total lipids in tonoplast from oat mesophyll (Verhoek et al., 1983). These studies have undoubtedly underestimated the total sphingolipid content of plasma membrane and tonoplast because the abundant GIPC class was not included in measurements of total lipids due to difficulties in the extraction and isolation of these lipids (Markham et al., 2006). Because many of the sphingolipid biosynthetic enzymes have been localized to the ER, it is also presumed that this organelle is enriched in sphingolipids (Tamura et al., 2001; Hillig et al., 2003; Chen et al., 2006, 2008). With the recent reports of improved methods for sphingolipid extraction and mass spectral analysis, the comprehensive characterization of sphingolipid compositions of specific membranes should be forthcoming.

In addition, two recent studies have highlighted the importance of sphingolipids for the integrity of the endomembrane system. In these reports, *Arabidopsis thaliana* pollen was examined by electron microscopy to determine membrane defects associated with the loss of sphingolipids in plants deficient in sphingolipid synthesis due to knockout of the two *LCB2* genes of serine palmitoyltransferase (Dietrich et al., 2008; Teng et al., 2008). These studies revealed aberrant vesiculation of the ER, the absence of Golgi bodies, and the lack of the Golgi-derived intine layer on the periphery of the mutant pollen. Sphingolipids also play critical roles in endomembrane-associated processes in plant cells. For example, defects in endocytic membrane trafficking were observed in an *Arabidopsis thaliana* mutant with reduced content of very-long chain fatty acids in sphingolipids (Zheng et al., 2005). The results collectively show that sphingolipids are not only quantitatively important components of the endomembrane system in plants, but also are essential for the ontogeny and function (e.g., membrane trafficking) of endomembranes.

It is likely that alterations in sphingolipid content and composition can significantly impact the physical properties of plasma membrane and tonoplast, given the abundance of sphingolipids in these membranes. Because the plasma membrane and tonoplast are of primary importance for osmotic adaptation and ion fluxes, changes in sphingolipid content and composition almost certainly impact the ability of plants to respond to stresses, such as drought, freezing, salt, and heavy metals. Indeed, GlcCer content of plasma membrane has been shown to decrease in a number of plant species during the events of cold acclimation that lead to enhanced freezing tolerance (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994; Uemura et al., 1995). In addition, enrichment of the *cis*-Δ8 isomer of t18:1 in LCBs of *Arabidopsis thaliana* sphingolipids was recently shown to increase aluminum tolerance (Ryan et al., 2007). With the availability of mutants and the identification of genes for key sphingolipid metabolic enzymes, researchers are now in a position to dissect the roles of specific structural features of sphingolipids in environmental stress resistance in order to rationally engineer desirable compositions of plasma membrane and tonoplast for improved crop productivity.

### *2 Physicochemical Behavior of Sphingolipids in Aqueous Solutions and Microdomain Formation*

The biological properties of sphingolipids are derived from their amphiphilic nature and their behavior in aqueous solutions. Since biological membranes are highly organized complex mixtures

of hundreds of lipid and protein species in which the performance of individual lipids is difficult to evaluate, most of the knowledge in this field has been obtained from studies of model membranes. LCBs, such as d18:1Δ4*trans*, tend to form micelles in aqueous environments, showing similar critical micellar concentration values (6–112 μM) to other single chain amphiphiles, such as lysophospholipids and aliphatic-type detergents (Le Stunff et al., 2002c; Garmy et al., 2005). The ability to form micelles is important for the signaling role of these compounds, although there is low probability that LCBs physically leave the membrane environment where they are generated. Complex sphingolipids in aqueous solutions have the tendency to associate in complex, self-organized structures, such as liposomes and bilayers that can serve as model systems for biological membranes. Their polar and non-polar structural moieties contribute to this formation. The aliphatic carbon chains of the fatty acid and the LCB moieties contribute to the high melting temperatures ($T_m$) of sphingolipids, and promote the hydrophobic effect that spontaneously and cooperatively maintains them together with other membrane lipids, giving them a relatively rigid conformation in the membrane (Pascher, 1976; Pascher and Sundell, 1977; Ramstedt and Slotte, 2006). The hydrophilic head groups of sphingolipids permit lipid–lipid or lipid–protein interactions that either reinforce or decrease the non-polar cohesion of the hydrophobic tails, since phosphate and carbohydrate groups may introduce hydrogen bonding or steric and coulombic repulsions with neighboring molecules (Brown, 1998).

Sphingolipids tend to segregate with sterols in very highly packed and ordered membrane regions, in a phase characterized as $L_o$ (liquid-ordered phase) (Mayor and Rao, 2004; Simons and Vaz, 2004). $L_o$ is an ordered, solid-like gel state that is different to the $L_d$ (liquid-disordered phase) or less ordered fluid liquid crystalline state that most of the membrane glycerolipids adopt (Ohvo-Rekilä et al., 2002). The spontaneous and favored association between sterols and sphingolipids seems to be promoted by three factors: (1) the effective length of the rigid sterol ring system that is equivalent to a 17-carbon all *trans* hydrocarbon chain, matching the LCB extension (Wu and Chi, 1991; Brown, 1998); (2) the establishment of a hydrogen bond between the 3-β–OH group of the sterol and the amide group of the ceramide (Curatolo, 1987; Massey, 2001); and (3) the limited contribution of the sterol polar head group to steric hindrance with head groups of adjacent lipids (Xu et al., 2001; London, 2002).

Most of the membrane lipids are in the $L_d$ phase, which is fluid and laterally homogenous in terms of lipid and protein composition. Sphingolipids and sterols become transiently associated to form an $L_o$ phase, a physical region that moves together as a patch or "lipid raft" with free lateral mobility in the $L_d$ phase lipids (Xu et al., 2001; Ohvo-Rekilä et al., 2002; Binder et al., 2003) (Fig. 6). The size, half-life, and formation dynamics of these membrane domains are not well understood. Data available from animal systems suggest that microdomains may constitute membrane structured regions ranging from 10 to 100 nm, perhaps containing from 100 to 100,000 lipid molecules (Simons and Tomre, 2000). The microdomains can fuse to form wider structures resembling membrane platforms for which cellular residence time varies from milliseconds to hours (Simons and Tomre, 2000; Binder et al., 2003; Ramstedt and Slotte, 2006). It is not clear whether some proteins facilitate lipid association or if the lipids promote the specific association of proteins in order to form functional microdomains. The existence of lipid rafts or microdomains does not contradict the essence of the fluid mosaic model for membrane structure, but rather expands upon it (Edidin, 2003; Vereb et al., 2003). In this regard, the lipids in the $L_d$ phase not only possess individual motion consistent with the Singer and Nicolson model (Singer and Nicolson, 1972), but also the lipids in the $L_o$ phase that form the microdomain undergo free lateral diffusion as a patch in the $L_d$ phase behave as a raft (Fig. 6).

Lipid rafts or microdomains possess structural characteristics that accomplish specific cellular functions. In yeast and animal cells, lipid rafts may act as supramolecular platforms to recruit proteins by favoring efficient physiological interactions. The establishment of favorable molecular interactions is critical to membrane processes such as signaling, polarity, and trafficking, responses to biotic and abiotic stresses, and cell specialization and cell maintenance (Hearn et al., 2003; Hering et al., 2003; Schuck and Simons, 2004). Lipid microdomains have also received extensive

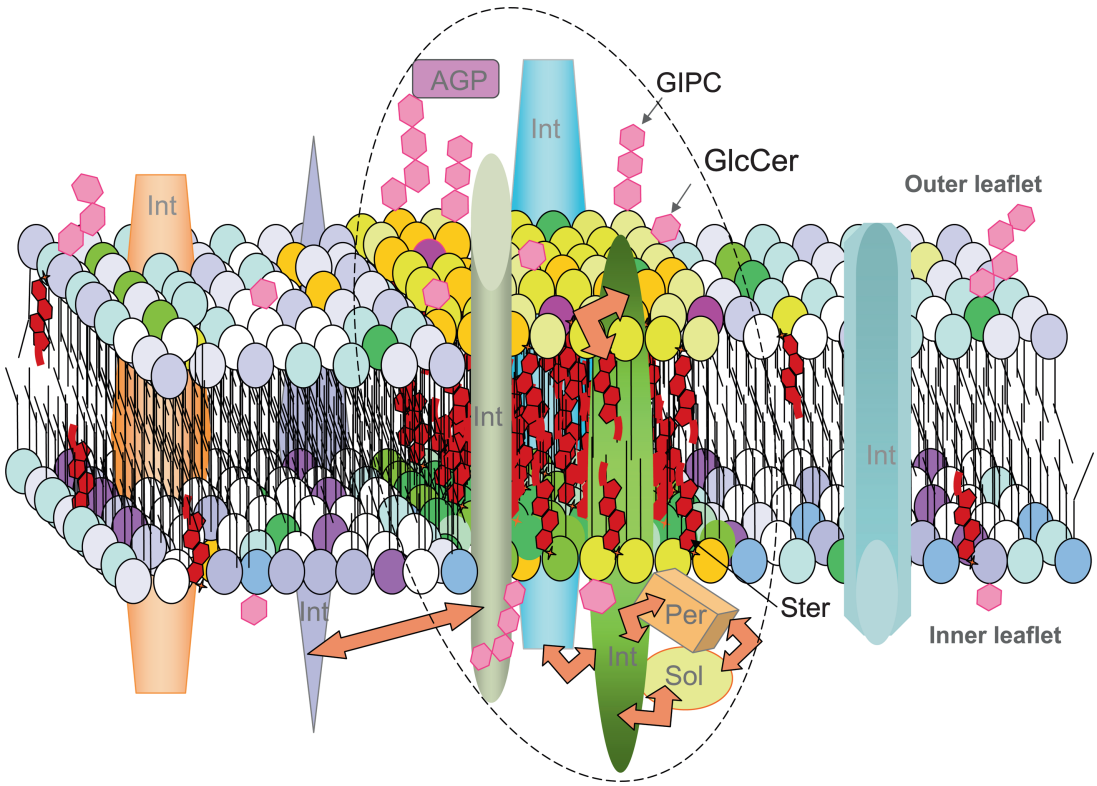


*Fig. 6.* Sphingolipids as structural building blocks of membrane microdomains or lipid rafts. Sphingolipids, including glucosylceramides (GlcCers) and glycosyl inositolphosphoceramides (GIPC), can spontaneously associate with high affinity to sterols (Ster) in cell membranes, forming transient domains that provide a site where specific interactions between lipid and/or proteins are favored (the microdomain is indicated by circled area of membrane). However, these assembly sites may function to exclude proteins in order to avoid undesirable functional collisions as well. *Arrows* indicate favored or unfavored interactions of proteins. Integral membrane proteins (Int), arabinogalactan proteins (AGP), peripheral (Per) and soluble (Sol) proteins are representative proteins associated to these regions (illustration by Laura Carmona-Salazar) (See Color Fig. 5 on Color Plate 3).

attention with regard to the role of rafts as portals for infection by viral, bacterial, and protozoan pathogens (van der Goot and Harder, 2001; Mañes et al., 2003; Lafont et al., 2004; Riethmuller et al., 2006).

### *3 Sphingolipid-Rich Microdomains in Plant Membranes*

Evidence for microdomain formation in plants and in other eukaryotes comes from the isolation of detergent resistant membranes (DRMs) or detergent insoluble membranes, which are obtained from membranes treated with detergents at 4°C. Given the highly cohesive hydrophobic and hydrophilic interactions among sterols and sphingolipids, these regions are difficult to dissolve and can be isolated for biochemical studies (Schroeder et al., 1994). Whether these preparations actually constitute the microdomains present at the membrane or are just collected components of the original membrane structures is a controversial matter (Schroeder et al., 1994; Simons and Ikonen, 1997; Munro, 2003). However, to date, DRMs are the best biochemical method for studying the composition of lipid rafts and their cell function (Lingwood and Simons, 2007).

Membrane microdomains were recognized and intensively studied in animal cells at least 12 years earlier (Simons and Ikonen, 1997; Brown and London, 1998). Peskan et al. (2000) obtained the first preparation that suggested the existence of membrane microdomains in plants. Since then, DRM fractions have been isolated from plasma membranes, Golgi apparatus, or microsomes from a number of different plant sources

(Mongrand et al., 2004; Shahollari et al., 2004; Borner et al., 2005; Laloi et al., 2007; Lefebvre et al., 2007). This distribution is the result of the synthesis and assembly of the raft components in the endoplasmic reticulum and Golgi apparatus, and the delivery to the plasma membrane or other target membranes by vesicle trafficking routes (Futerman, 2006).

Free sterols and sphingolipids are enriched in DRMs and respectively constitute approximately 30% and 20–40% of the total lipid content of DRMs (Mongrand et al., 2004; Laloi et al., 2007; Lefebvre et al., 2007). Most studies to date have reported only GlcCers in plant-derived DRMs and have overlooked the more abundant GIPCs, as these molecules possess a high degree of polarity and are not extracted with solvent systems that have been traditionally used in plant lipidology. As a result, reports describing the lipid content of DRMs from plants have almost certainly underestimated levels of sphingolipids. Nevertheless, the enrichment of sphingolipids in DRMs obtained from different membrane sources ranges from 1.3- to 5-fold relative to the sphingolipid content of the bulk membranes, and sphingolipids with trihydroxy and dihydroxy LCBs are found in plasma membranes and isolated DRM (Mongrand et al., 2004; Borner et al., 2005; Laloi et al., 2007; Lefebvre et al., 2007).

Proteins are also major components of plant lipid rafts. The number of identified proteins in DRM varies from 15 to 270, depending on the sensitivity and resolution of the methods employed (Mongrand et al., 2004; Borner et al., 2005; Lefebvre et al., 2007). DRM proteins fall into several groups related to their possible function: (1) signaling proteins, such as leucine-rich repeat receptor-like kinases, serine/threonine kinases, calcium-dependent kinases, small GTP binding proteins, glycosylphosphatidylinositol (GPI) anchored proteins and proteins responsive to pathogen elicitors and those involved in defense responses to biotic and abiotic stress; (2) transmembrane transport proteins, such as proton pumps, water channels, anion and cation carriers, and putative plant hormone transporters; (3) intracellular trafficking proteins, such as cytoskeletal proteins and proteins characteristic of secretory vesicles or trafficking assembly complexes; and (4) metabolic enzymes, especially those involved in the synthesis of cell wall polymers (Peskan et al., 2000; Mongrand et al., 2004; Shahollari et al., 2004; Borner et al., 2005; Morel et al., 2006; Lefebvre et al., 2007). Recently, an E3 ligase, a protein possibly involved in fumonisin $B_1$ induction of programmed cell death has been reported in DRMs from *Arabidopsis thaliana* (Lin et al., 2008).

The interaction of sphingolipids with membrane proteins has been studied. Biochemical experiments, supported by several crystallographic structures of membrane proteins, have shown that the surrounding lipids influence the activity of membrane proteins (Camara-Artigas et al., 2002; Long et al., 2007). Like most membrane lipids, sphingolipids may affect protein activity by modifying protein conformations. As essential components of membrane microdomains, sphingolipids may contribute to protein function by facilitating protein–protein interactions required for biological activity. Given the types of proteins found in microdomains (Borner et al., 2005), alterations in the content and composition of sphingolipids likely impacts important cellular activities, such as synthesis and degradation of cell walls and intercellular movement of plant hormones.

Membrane thickness in microdomains is greater than in non-microdomains, due to the presence of longer and more extended hydrophobic chains. This suggests that integral proteins must match their hydrophobic residues to reside in either of these regions in such a way that migration to another region brings about conformational changes necessary for protein secretion (Allende et al., 2004). It has been hypothesized that the carbohydrate moiety of sphingolipid head groups may interact with an aromatic residue of a sphingolipid-binding domain consisting of a hairpin structure that stabilizes the lipid–protein interaction (Fantini, 2003).

What is the role of membrane microdomains in plant cells? Experiments in plant and animal cells have revealed microdomains as sites for pathogen entry, and have identified molecular components that may give some clues to function (Mañes et al., 2003; Bhat et al., 2005; Grennan, 2007). Many of the proteins identified in microdomains suggest a role as metabolic or secretion centers for housekeeping activities. They could also be sites where the recognition of external signals and their initial transduction takes place.

Special attention must be given to the recruitment of signaling proteins involved in defense responses to pathogens. It is thought that specific and optimized lipid microenvironments for protein–protein interactions mediate these events in plants, resulting in an efficient and rapid response to a metabolic demand or to a stress situation, as demonstrated for yeast and animal systems (Bagnat et al., 2000; Hering et al., 2003). The same phenomena could be occurring in cases where polarization of membrane components is required, such as pollen tube formation. In addition to proteins, microdomain lipids may participate in these functions. Based on recent reports that LCBs, in their free or phosphorylated form, act as mediators in signal transduction pathways (Ng et al., 2001; Coursol et al., 2003), a role for membrane microdomains as a sphingolipid signaling reservoir can also be envisioned.

## B Sphingolipids as Signaling Molecules

In contrast to the large amount of information available from animals and yeast, relatively little is known about the signaling properties of sphingolipids in plants (Worrall et al., 2003). Recently, several reports have documented bioactive functions of sphingolipids in drought stress, programmed cell death, and pathogenesis (Fig. 7).

### 1 Role of Sphingolipids in Drought Stress Signaling and Regulation of Stomatal Closure

LCBs play a role in signaling in plant cells by mediating stomatal closure. Stomata are pores in the epidermis of leaves and stems that are important for carbon dioxide uptake during photosynthesis and for the reduction of water loss by transpiration. Guard cells regulate stomatal

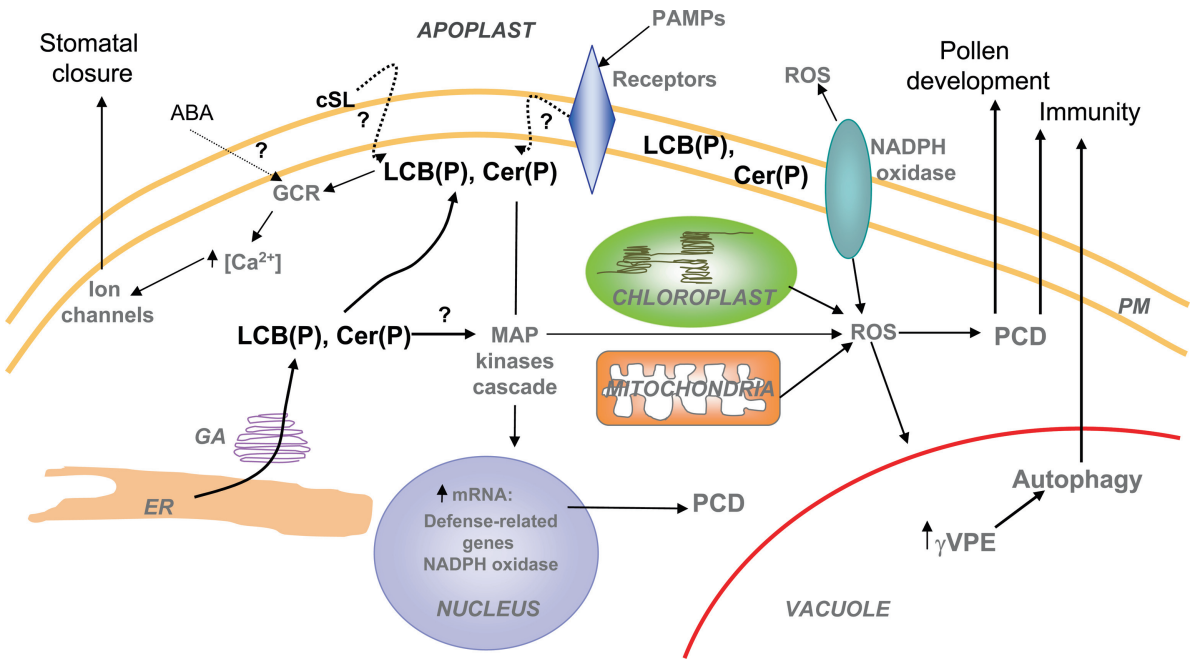


*Fig. 7.* Bioactive roles of sphingolipid metabolites. Free or phosphorylated long chain bases (LCB(P)) and ceramides (Cer(P)) are intracellular mediators in transduction pathways that lead to control of stomata closure and programmed cell death. Given their demonstrated role in the generation of reactive oxygen species (ROS) and the initiation of programmed cell death (PCD), it is possible that these sphingolipid metabolites also contribute to pollen development and immunity to pathogens. With regard to plant–pathogen interactions, sphingolipid metabolites may be associated with pathogen-induced triggering of MAP kinases cascades and may serve as downstream components of signal transduction pathways mediated by pathogen-associated molecular patterns. It is unknown what the relative contributions of de novo sphingolipid synthesis and sphingolipid degradation are to the production of bioactive LCBs and ceramides. Abbreviatons: cSL, complex sphingolipids; ER, endoplasmic reticulum; GA, Golgi apparatus; PM, plasma membrane; GCR, G-protein coupled receptor; PAMPs, pathogen-associated molecular patterns; γVPE, vacuolar processing enzyme (See Color Fig. 6 on Color Plate 4).

opening and closure by responding to diverse environmental and chemical cues, such as light intensity, temperature, water status, $CO_2$ levels and hormonal signals. The plant hormone ABA regulates stomatal opening and closure by elevating cytosolic $Ca^{2+}$ levels, which, in turn, activates plasma membrane anion channels in guard cells. The resulting $K^+$ efflux causes loss of turgor and stomatal closing (Schroeder et al., 2001). The role of LCBs in this ABA-mediated signaling cascade was first shown as a 2.4-fold increase in sphingosine (d18:1Δ4*trans*)-1-phosphate (S1P) levels in drought-treated *Commelina communis* plants compared to control plants. Exogenously applied S1P induces stomatal closure at low concentrations (4–6 μM), while the 1-phosphate ester of d18:0 does not show this effect. The transient increase in cytosolic $Ca^{2+}$ concentration appears to be the link between S1P and the ABA-mediated signal transduction pathway leading to ion channel activation (Ng et al., 2001). Further evidence for the involvement of S1P and phytosphingosine-1P was obtained by direct assay of LCB kinase activity in mesophyll and guard cell protoplasts in *Arabidopsis thaliana* leaves (Coursol et al., 2003, 2005). Treatment of *Arabidopsis thaliana* plants with ABA was found to activate the LCB kinase in vivo, and this activity was sensitive to *N*,*N*-dimethylsphingosine, a known inhibitor of mammalian LCB kinases (Coursol et al., 2003). The target of S1P in animal cells is a G-protein coupled receptor, which in turn interacts with a transmembrane G-protein. G-proteins are a family of signal-transducing proteins consisting of multiple subunits. The involvement of G-proteins in the signal-transduction pathway for S1P in plants was also suggested by Coursol and co-workers (Coursol et al., 2003), who showed that *Arabidopsis thaliana* mutants lacking the G-protein α-subunit were unable to respond to exogenously applied S1P (Fig. 7).

### *2 Sphingolipid-Associated Programmed Cell Death and Autophagy*

Programmed cell death is an essential event in plant development and stress responses, sharing some features with apoptosis in animal cells (van Door and Woltering, 2005). The pharmacological approach of adding exogenous LCBs and ceramides to plant cell cultures and tissues has supported the role of these molecules as potential mediators of programmed cell death. Synthetic C2 ceramide (50 μM) induces hydrogen peroxide production and cell death in *Arabidopsis thaliana* cell culture in a calcium-dependent manner. Cytosolic $Ca^{2+}$ increases about twofold a few seconds after ceramide addition and its role in promoting cell death downstream is shown by the effect of $La^{3+}$, a calcium channel inhibitor (Townley et al., 2005). Free LCBs have similar effects and appear to be more active. The application of d18:1Δ4*trans*, d18:0, or t18:0 at a concentration of 2 μM to *Arabidopsis thaliana* leaves causes an accumulation of reactive oxygen intermediates and cell death (Shi et al., 2007). In contrast, the phosphorylated intermediates of the free LCBs do not have this activity and even prevent cell death when they are applied simultaneously with the non-phosphorylated form. Interestingly, this protective effect shows a structural specificity, as each phosphorylated LCB was only able to block programmed cell death initiated by its corresponding free LCB (Shi et al., 2007), suggesting that the ratio of specific phosphorylated and non-phosphorylated LCBs might act as cell death regulators.

A possible control mechanism could be achieved through autophagy, an event necessary for protein and organelle degradation. Macroautophagy, usually referred to as simply autophagy, is a conserved mechanism in eukaryotic cells. This process requires the formation of double membrane vesicles that engulf cytoplasmic contents and sometimes organelles. Once these vesicles are acidified, their external membranes fuse with lysosomes (in animals) or the vacuole (in plants), and the vesicles with only inner membranes are introduced into these structures to be degraded through proteolysis (Klionsky, 2005).

Recently, it has been shown that autophagy is a crucial process in initiating the hypersensitive response of programmed cell death, and also in preventing programmed cell death during innate immunity. In *Nicotiana benthamiana*, disruption of the *BECLIN1* gene, the ortholog of conserved mammalian and yeast tumor suppressor genes, causes unrestricted local and systemic cell death upon pathogen challenge. *BECLIN1*-silenced plants show a reduction of double-membrane vesicles in the vacuole (Liu et al., 2005). The exact role of the BECLIN1 protein in regulat-

ing autophagy is not known. However, in mammalian cells, enhanced levels of BECLIN1 are associated with ceramide-mediated autophagy (Scarlatti et al., 2004). S1P induces autophagy in animal cells, but in contrast to ceramide-induced autophagy, BECLIN1 protein levels are not modified (Lavieu et al., 2006).

### *3 Sphingolipids in Plant–Pathogen Interactions*

Plants recognize a wide array of microbial-derived molecules known as pathogen-associated molecular patterns through specific molecular interactions with the products of plant resistance genes (Jones and Dangl, 2006). This interaction initiates a response by the plant to arrest pathogen colonization. When this response is particularly accelerated and amplified, the reaction is termed a hypersensitive response (HR). Lipid-derived molecules, such as jasmonic acid, have been implicated in plant defense signaling (Shah, 2005). Recent evidence also implicates sphingolipids as potential signal molecules in plant defense pathways. This hypothesis is based on the biological activity of AAL toxin and fumonisin $B_1$, which are produced by necrotrophic plant pathogenic fungi. Both toxins are sphingosine-analog mycotoxins that disrupt sphingolipid synthesis as they target the acyl-CoA-dependent ceramide synthase (Fig. 3). AAL toxin is a known pathogenicity factor required for *Alternaria alternata* f. sp. *lycopersici* infection of tomato (Gilchrist and Grogan, 1976). Fumonisins are required for the development of foliar disease symptoms produced by *Fusarium verticillioides* infection (Glenn et al., 2008). As inhibitors of the acyl-CoA-dependent ceramide synthase, these toxins cause a marked increase of free LCBs. In *Lemna pausicostata* leaf discs, 1 μM fumonisin $B_1$ and 1 μM AAL toxin produce a 75- and a 129-fold increase in sphinganine levels, respectively (Abbas et al., 1994). In addition to augmenting LCB levels, both toxins produce cell death in various plant species (van Asch et al., 1992; Abbas et al., 1994; Stone et al., 2000). Fumonisin $B_1$-induced cell death in *Arabidopsis thaliana* has a number of features in common with HR, including the presence of localized lesions, reactive oxygen species production, and defense gene expression that depend on functional salicylic acid, ethylene and jasmonic acid-mediated pathways (Asai et al., 2000) (Fig. 7).

Resistance to these toxins has shed some light on the potential mechanisms leading to programmed cell death by sphingolipids. In tomato, the *Asc-1* gene confers resistance to AAL toxin and to the fungus *Alternaria alternate* f. sp. *lycopersici*. When homozygous tomato genotypes harboring the resistance gene are treated with AAL toxin, they do not accumulate the LCBs d18:0 and t18:0 to the same high levels as the susceptible *asc/asc* mutant (Abbas et al., 1994). In addition, the AAL toxin-resistant plants are able to synthesize complex sphingolipids, and cell death is not observed (Spassieva et al., 2002). Overexpression of *Asc-1* confers resistance to AAL toxin and *Alternaria alternata* in susceptible tomato genotypes and in *Nicotiana umbratica* (Brandwagt et al., 2002). The Asc-1 polypeptide is homologous to the *Saccharomyces cerevisiae LAG1*-encoded ceramide synthase (Spassieva et al., 2002), and its overexpression likely reduces the accumulation of cytotoxic LCBs.

Characterization of the *Arabidopsis thaliana* fumonisin $B_1$-resistant mutant *fbr11-1* provided direct evidence that the accumulation of free LCBs mediates cell death (Shi et al., 2007). In this mutant, the gene for the LCB1 subunit of serine palmitoyltransferase harbors a T-DNA insertion in its 3′ untranslated region leading to a reduced transcript level compared to wild type plants. Basal levels of LCBs are not altered in these plants, but the mutants are unable to achieve high levels of LCBs after treatment with fumonisin $B_1$ and are, therefore, unable to initiate the cell death response (Shi et al., 2007). The *fbr11-1* mutants do not accumulate superoxide anion and hydrogen peroxide when treated with fumonisin $B_1$, suggesting that reactive oxygen species mediate LCB-induced cell death. The fact that both AAL toxin-resistant tomato genotypes and fumonisin $B_1$-resistant *Arabidopsis thaliana* mutants accumulate LCBs upon treatment with mycotoxin, but to a lesser degree than the susceptible genotypes, suggests that a threshold level of free LCBs must be reached to trigger programmed cell death.

Other fumonisin $B_1$-resistant *Arabidopsis thaliana* mutants are available to study the transduction pathway, which, triggered by fumonisin $B_1$ and accompanied by increases in levels of LCBs, leads to programmed cell death. The *fbr6* mutant

survives and develops in the presence of fumonisin $B_1$ but shows an elongated petiole and exaggerated leaf margin serration in the absence of the toxin (Stone et al., 2005). The FBR6 polypeptide was identified as a putative transcriptional regulator (AtSPL14) with a highly conserved DNA binding domain (GTAC), nuclear localization signal and an ankyrin protein interaction motif. FBR6 belongs to the SQUAMOSA promoter binding protein family that have been linked to plant growth and development (Liang et al., 2008). Indeed, the *fbr6* mutant shows elongated petioles and enhanced leaf margin serration (Stone et al., 2005). Although the role of FBR6 in fumonisin $B_1$ resistance is not yet clear, identification of its target genes might help to explain this function. Other fumonisin $B_1$ resistant mutants identified to date include *fbr1* and *fbr2* (Stone et al., 2000). In the presence of 1 μM fumonisin $B_1$, *fbr1* and *fbr2* plants develop normally and show reduced levels of the plant defensin gene transcript *PDF1.2* when compared to wild type plants. Although levels of other resistance gene transcripts are not modified, these mutants show increased resistance to a virulent strain of *Pseudomonas syringae* pv. *maculicola* (Stone et al., 2000). Identification of the *FBR1* and *FBR2* gene products should provide further clarification of their potential role in sphingolipid metabolism.

Two *Arabidopsis thaliana* mutants with an accelerated cell death (acd) phenotype provide further evidence linking sphingolipid metabolism to the hypersensitive response. Mutants *acd-11*constitutively express cell death and HR-related genes and accumulate phytoalexins and callose. The *ACD11*-encoded polypeptide is related to a mammalian glycolipid transfer protein, and in vitro experiments have shown that the recombinant protein can transport sphingosine (d18:1) between membranes with high binding affinity (Brodersen et al., 2002). A second mutant, *acd5*, has a similar phenotype and develops normally but shows unrestricted cell death when infected by *Pseudomonas syringae*, in a salicylic acid-dependent pathway (Greenberg et al., 2000). The ACD5 protein has ceramide kinase activity and a balance between its product, ceramide-1-phosphate and ceramide might trigger the cell death program (Liang et al., 2003). As it is the case with LCB-induced programmed cell death, phosphorylation of ceramides appears to be a mechanism for reducing the potential cytotoxic effects of accumulated ceramides.

A critical step in the fumonisin-induced cell death pathway is protease activation of the vacuolar processing enzymes (VPE), a family of cysteine proteases responsible for the maturation of some proteins, which are found in both developing seed and vegetative tissues (Kuroyanagi et al., 2005). The vegetative forms are induced by wounding and senescence, and are thought to be involved in programmed cell death. Genetic evidence supporting the role of these enzymes in plant cell death comes from an *Arabidopsis thaliana* null mutant, which lacks all four VPE genes. In the VPE mutant, vacuoles do not collapse in fumonisin $B_1$-treated leaves, whereas wild type leaves show vacuole disruption and subsequent cell death (Kuroyanagi et al., 2005).

In addition, a sphingolipid-mediated signaling cascade involving sphingolipid-specific receptors, protein kinases, and GTPases has been identified in interactions between rice and a pathogenic fungus. In this regard, two classes of GlcCers ("Cerebrosides A and C") isolated from the rice pathogen *Magnaporthe grisea* (rice blast) have been shown to elicit HR-type cell death and the accumulation of the phytoalexin momilactone when applied to rice leaves (Koga et al., 1998). These responses ultimately promote resistance to rice blast and other pathogens. The elicitation could also be conferred by ceramides generated from Cerebrosides A and C, but fatty acid and LCB degradation products of these GlcCers or mammalian GlcCers were unable to elicit this response in rice, suggesting the presence of a receptor in rice that is able to specifically recognize fungal sphingolipids (Koga et al., 1998; Umemura et al., 2000). In addition, rice containing a mutation in the G protein α subunit displayed reduced response to the *Magnaporthe grisea* Cerebroside A and C elicitors, as measured by $H_2O_2$ accumulation and pathogenesis-related (PR) gene expression, and lacked resistance to rice blast (Suharsono et al., 2002). Resistance to rice blast could be restored in rice with the mutant heteromeric G protein α-subunit by overexpression of OsRac1, a homolog of RacGTPase and an important regulator of cell death and disease resistance in rice (Suharsono et al., 2002). This also restored $H_2O_2$ production and expression of PR genes in the host plants. Moreover, silenc-

ing of *OsRac1* resulted in reduced levels of the OsMAPK6 protein, a mitogen-activated protein kinase, and also lead to reduced activation of OsMAPK6 by the GlcCer elicitor (Lieberherr et al., 2005). These results are consistent with a sphingolipid-induced cascade that involves transmission of a signal from a sphingolipid receptor in rice to the heteromeric G protein α-subunit and then to the downstream OsRac1 and OsMAPK6 that triggers the production of reactive oxygen species and PR gene expression and ultimately disease resistance in the host plant (Suharsono et al., 2002; Lieberherr et al., 2005).

### *C Relevance of Sphingolipids to Chloroplasts and Photosynthesis*

Although sphingolipids have not been previously identified in chloroplast membranes, the biosynthesis of sphingolipid long-chain bases is directly linked to metabolic reactions that occur in chloroplasts. In this regard, the palmitoyl-CoA precursor of LCBs is formed by de novo fatty acid synthesis in chloroplasts. This substrate of serine palmitoyltransferase (SPT) arises from the release of palmitic acid from acyl carrier protein (ACP) by palmitoyl-ACP thioesterase or fatty acid thioesterase (FATB) for export to the cytosol. It has been shown that null mutants for *FATB* display reduced growth (Bonaventure et al., 2003). The basis for this phenotype is not clear given that no differences were detected in the content of waxes and sphingolipids on a dry weight basis between the wild type and mutant (Bonaventure et al., 2003). One possibility is that the reduced growth results from restricted synthesis of sphingolipids due to the reduced availability of the palmitoyl-CoA substrate. In fact, the phenotype of these mutants is similar to that of *Arabidopsis thaliana* lines with partial RNAi suppression of the LCB1 subunit of serine palmitoyltransferase (Chen et al., 2006). Like the *FATB* mutants, the *LCB1* RNAi lines do not have reduced sphingolipid content on a dry weight basis (Chen et al., 2006). An interpretation of this result is that plants adjust their growth based on the availability of sphingolipids, which can be determined by the pool sizes of the palmitoyl-CoA precursor or by the activity of serine palmitoyltransferase. As such, fatty acid biosynthetic reactions, including the release of palmitic acid from ACP, in chloroplasts can play a key role in regulating sphingolipid synthesis in the ER. This, in turn, impacts the growth and physiology of the plant.

In addition, it has recently been shown that complete or partial loss of LCB C-4 hydroxylation results in up to a three-fold increase in total sphingolipid content in *Arabidopsis thaliana* leaves (Chen et al., 2008). Lipidomic analyses conducted on these plants revealed decreases in levels of principally chloroplast-specific lipids, monogalactosyldiacylglycerol and phosphatidylglycerol, relative to the wild-type controls (Chen et al., 2008). These changes were largely accounted for by reductions in molecular species that contain C16 fatty acids. One explanation for the altered content of monogalactosyldiacylglycerol and phosphatidylglycerol in the C-4 hydroxylase deficient plants is that the increased demand for palmitoyl-CoA to support enhanced sphingolipid synthesis is compensated for by reductions in the flux of palmitic acid into chloroplast lipids. The examples of the FATB and LCB C-4 hydroxylase mutants illustrate the interdependency of lipid metabolic pathways in the chloroplast and ER.

Furthermore, several lines of evidence point to a relationship between sphingolipid function and chloroplasts. It has been shown that disruption of sphingolipid synthesis by application of fumonisin $B_1$ results in light-dependent cell death in leaves of jimsonweed (*Datura stratonium* L.) (Abbas et al., 1992) and *Arabidopsis thaliana* (Stone et al., 2000). In the case of jimsonweed, chloroplast membrane integrity is lost in response to fumonisin $B_1$ treatment, as evidenced by the destruction of the chloroplast outer envelope, disorganization of thylakoid membranes, and large reductions in chlorophyll content (Abbas et al., 1992). Fumonisin $B_1$-associated programmed cell death appears to be closely linked to the generation of reactive oxygen species (Asai et al., 2000; Shi et al., 2007). HR-related programmed cell death, such as that observed in *ACD5* and *ACD11* mutants, also appears to be mediated through reactive oxygen species (Greenberg et al., 2000; Brodersen et al., 2002; Liang et al., 2003). In the latter case, reactive oxygen species appear to be the product of light-dependent reactions in chloroplasts (Liu et al., 2007).

## VI Concluding Remarks

The study of plant sphingolipids has advanced remarkably during the past 10 years. Methods for the quantitative extraction and comprehensive analysis of plant sphingolipids are now in place, and many of the genes that are involved in sphingolipid synthesis have been identified and functionally characterized. In addition, it is now established that sphingolipids are essential molecules in plants and are major structural components of plasma membrane, tonoplast, Golgi apparatus, and likely other endomembranes. As membrane constituents, sphingolipids have been implicated in biotic and abiotic stress resistance, membrane transport, and the organization of membrane microdomains. It has also become evident that sphingolipid-related molecules are important regulators of cellular processes. The best characterized of these functions is the role of LCBs and likely ceramides as initiators of programmed cell death. Despite this progress, much remains to be learned about sphingolipid metabolism and function in plants. The important areas for future study include: (1) understanding how sphingolipid synthesis is regulated, (2) unraveling the enzymes of sphingolipid catabolism and how they function to mediate sphingolipid levels and the production of signaling molecules, (3) determining how sphingolipids are transported within the cells and function in endomembrane trafficking, (4) understanding the precise roles of sphingolipids in signaling pathways, and (5) obtaining a more complete understanding of the contributions of sphingolipids to membrane microdomain formation and function. In addition, with the availability of mutants for enzymes associated with the synthesis and modification of sphingolipids, researchers can now begin to address the functional basis for the immense structural diversity that is found in plant sphingolipids. A further challenge that remains is the development of a single, unified analytical platform for profiling of the content and composition of sphingolipids, glycerolipids, and sterols in plant organs and isolated membranes. With this capability, it will be possible to understand how alterations in one component of lipid metabolism impact the complete lipid metabolic network. With the momentum that has been gained in the study of plant sphingolipids, it is likely that many of these questions will be addressed in the coming decade.

## Acknowledgments

We thank Rebecca Cahoon for editing of the text. We also thank the Consejo Nacional de Ciencia y Tecnología (CONACYT), México (grant 55610) and Universidad Nacional Autónoma de México (UNAM) (grants DGAPA PAPIIT-IN207806 and PAIP 0690-2) for financial support to MGR, CONACYT (grant 50503Z) for financial support to JP, and the United States National Science Foundation (MCB-0312559, MCB-0843312) for financial support to EBC. MSG is supported by a fellowship from Consejo Nacional de Ciencia y Tecnología (CONACYT), México.

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