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Plant sphingolipids: function follows form

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Plant sphingolipids are structurally diverse molecules that are important as membrane components and bioactive molecules. An appreciation of the relationship between structural diversity and functional significance of plant sphingolipids is emerging through characterization of Arabidopsis mutants coupled with advanced analytical methods. It is increasingly apparent that modifications such as hydroxylation and desaturation of the sphingolipid nonpolar long-chain bases and fatty acids influence their metabolic routing to particular complex sphingolipid classes and their functions in signaling pathways and other cellular processes, such as membrane protein targeting. Here, we review recent reports investigating some of the more prevalent sphingolipid structural modifications and their functional importance in plants.

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Introduction

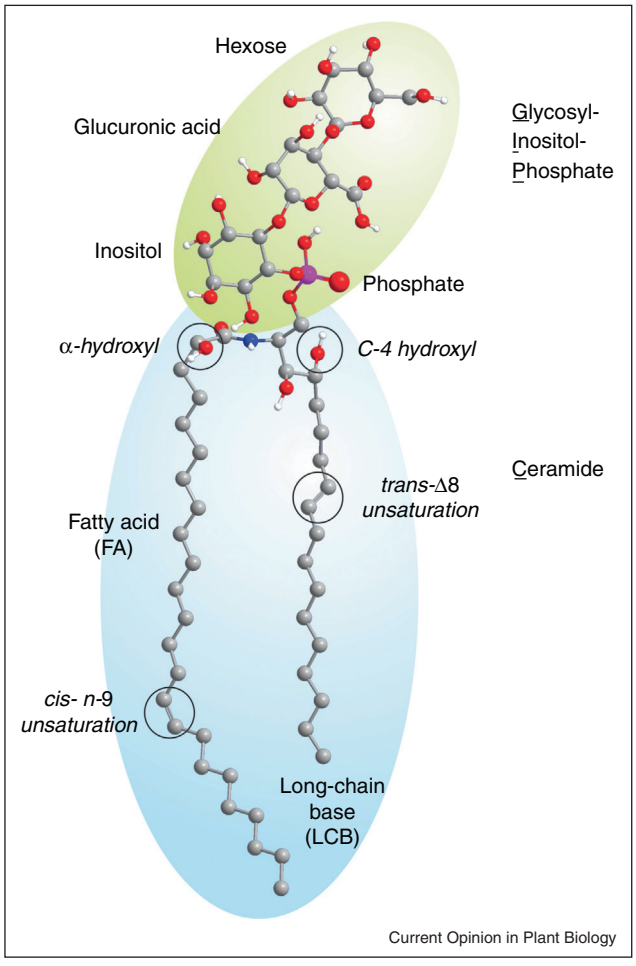
The metabolism and function of sphingolipids have gained increasing interest due to the recent appreciation of the quantitative significance of sphingolipids in specific membranes and their diverse roles in plant cells. Sphingolipids are ubiquitous in eukaryotes and have historically been studied in association with sphingolipid storage disorders such as Tay Sachs disease. In plants, sphingolipids are now recognized as major components of plasma membrane, tonoplast, and endomembranes. They exhibit substantial structural diversity, with hundreds of potential species, but until recently the significance of this structural complexity was unclear (Figure 1). Sphingolipids compose an estimated 40% of plasma membrane lipids and are enriched in the outer leaflet where they influence membrane integrity and ion permeability [1–3]. Sphingolipids are also intimately involved in endomembrane trafficking and are believed to function along with sterols and specific phospholipids in forming membrane domains (e.g. 'lipid rafts') [4–7]. Apart from their structural roles, sphingolipids are bioactive and participate in an array of processes and environmental responses such as programmed cell death (PCD) [8,9], pathogen-induced hypersensitive response (HR) [8,10•,11], ABA-dependent guard cell closure [12–14], host–pathogen interactions [15–17], and low-temperature signal transduction [18•,19,20]. Many of the structural modifications present in plant sphingolipids are distinct or absent from yeast and mammalian sphingolipids. Hence, the study of plant sphingolipids can provide insights into the importance of such structural diversity that is intractable in other eukaryotes. Mass spectrometry-based advances in analysis has provided a nearly complete 'sphingolipidome' of plant tissues [21] and the availability of Arabidopsis mutants has facilitated recent studies revealing the significance of sphingolipid structural complexity in controlling routes of metabolic flux and determining functional properties.

Sphingolipid structural complexity

Sphingolipid synthesis begins in the ER with condensation of serine and palmitoyl-CoA by serine palmitoyltransferase (SPT) (Figure 2) [22,23]. The product of this reaction is reduced to a C-1, C-3 dihydroxy C18 long-chain base (LCB), sphinganine, designated d18:0, indicating two hydroxyl groups (d), a chain length of 18-carbons (18), and no double bonds (0). Addition of a hydroxyl at C-4 produces the trihydroxy LCB, phytosphingosine or t18:0. LCBs may also be unsaturated by: (1) a *trans* double bond between C-4 and C-5 (or Δ4 position) of dihydroxy LCB and/or (2) a *cis* or *trans* double bond between C-8 and C-9 (or Δ8 position) of dihydroxy or trihydroxy LCB [24]. These structural variations mean that up to nine different LCB structures exist in plants. Both free LCBs and their C-1 phosphorylated derivatives (LCB-Ps) are present in plant cells at very low concentrations [21].

Additional sphingolipid structural complexity arises in the ceramide generated through amide linkage of a fatty acid (FA) to a LCB. The FA component of ceramide ranges in chain-length from C16 to C28, including odd-chain variants [24,25•], usually contain a C-2 or 'α-hydroxy' group, and can also contain a double bond at

Figure 1



A 3D, ball-and-stick representation of the predominant Arabidopsis sphingolipid highlighting structural modifications of particular interest (circles) discussed in the text. The glycosyl inositolphosphoceramide (GIPC) shown consists of a polar headgroup (enclosed in green) composed of phosphoinositol, presumably glucuronic acid based on structures from tobacco, and a final hexose. The precise conformation of the sugars and sugar linkages has yet to be determined for Arabidopsis. The ceramide portion (enclosed in blue) consists of a LCB that typically contains a C-4 hydroxyl and may contain a trans-Δ8 double bond (or unsaturation) linked via the nitrogen to a VLCFA, most commonly C24. The VLCFA of sphingolipids are mostly α-hydroxy fatty acids and in Arabidopsis, may contain a cis-n-9 unsaturation. Carbon atoms are shown in grey, oxygen in red, nitrogen in blue, phosphorous in purple and hydrogen in white: only polar hydrogen atoms are shown.

the n-9 position [26]. Similar to the LCB nomenclature, a saturated C24 fatty acid with α-hydroxylation is referred to as h24:0. The combination of 9 different LCBs and 32 different FAs yields 288 potential ceramides. Most (~90%) sphingolipids in plants are in a 'complex' form with a polar headgroup linked to C-1 of the LCB ceramide. The polar headgroup and non-polar ceramide give complex sphingolipids their amphipathic and bilayer-forming properties [21,27]. One complex sphingolipid, glucosylceramide (GlcCer), comprises ~30% of the sphingolipids in Arabidopsis leaves [21,27]. More abundant are the highly polar, anionic sphingolipids formed in the Golgi (Figure 2) with an inositolphosphate (IP) headgroup and up to 7 inositol-linked sugars collectively referred to as glycosyl inositolphosphoceramides (GIPCs) [21,28•,29]. In Arabidopsis, the most abundant GIPC, comprising ~60% of leaf sphingolipids, contains a hexose-hexuronic acid linked to IPC [21]. Although 288 different ceramides could be associated with each headgroup [21], GlcCers and GIPCs *in planta* have distinctive ceramide compositions: GlcCers are enriched in dihydroxy LCBs and C16 FAs whereas GIPCs are enriched in trihydroxy LCBs and very-long chain (≥C20) FAs (VLCFAs) [1,27]. The mechanism underlying this segregation is unknown, but it highlights the complex role that structure plays in the metabolic routing of sphingolipids.

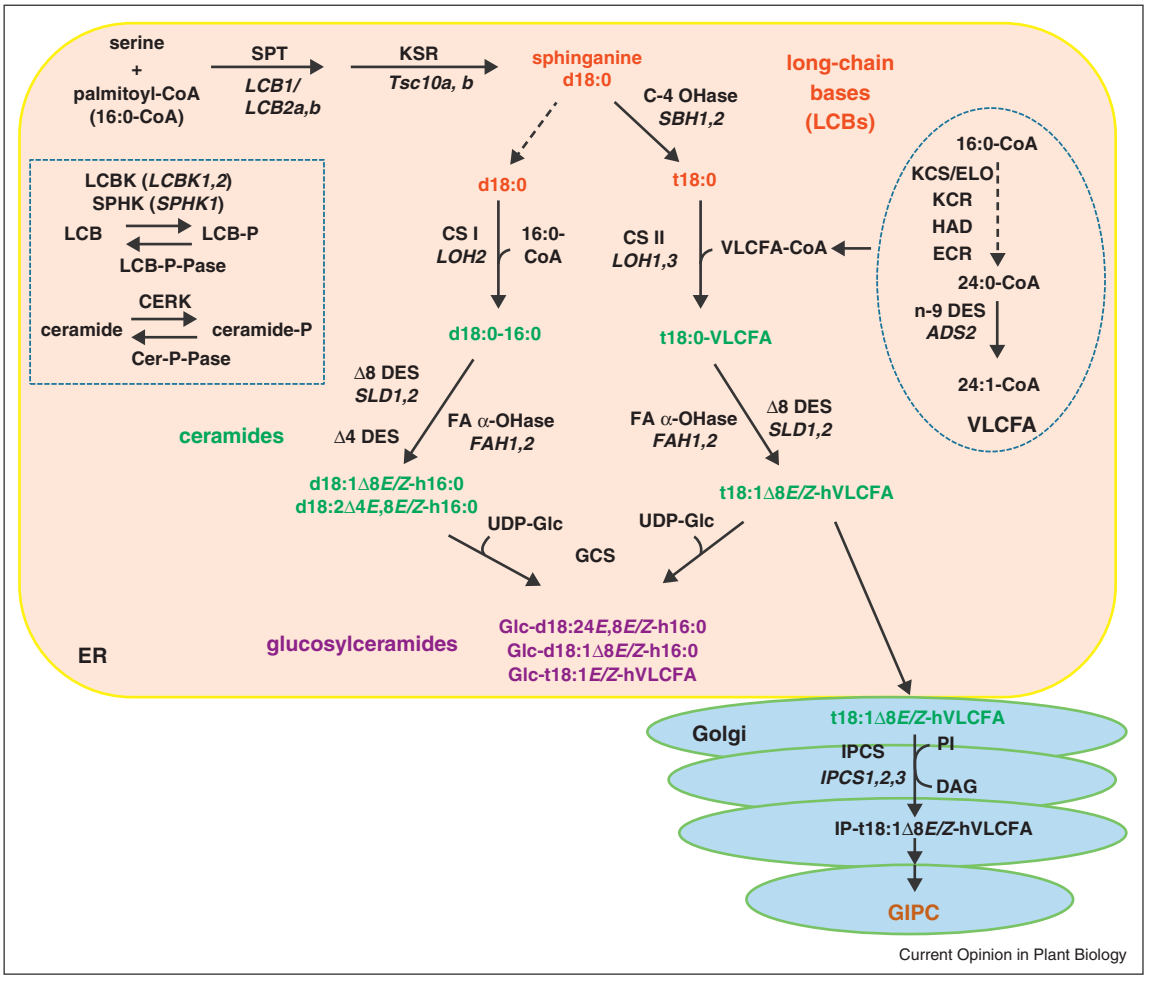
Significance of LCB and FA structures in sphingolipid metabolism and function

LCB hydroxylation and desaturation

The structural variability in plant LCBs arises from the activities of the LCB C-4 hydroxylase, Δ4 desaturase, and Δ8 desaturase (Figure 2). Recent characterization of Arabidopsis mutants lacking these enzymes has uncovered metabolic nuances of the sphingolipid biosynthetic pathway whereby LCB structure dictates downstream partitioning and ultimately determines the composition and content of total sphingolipids or specific sphingolipid classes [30••,31••,32]. These mutants have also allowed dissection of the significance of LCB hydroxylation and desaturation for plant growth and development.

In Arabidopsis, C-4 hydroxylation and Δ8 desaturation are quantitatively the most important, as ≥85% of the LCBs in leaf sphingolipids are trihydroxy as a result of the C-4 hydroxylase [encoded by *SBH1* (At1g69640) and *SBH2* (At1g14290)] [31••] and contain a *cis*-Δ8 or *trans*-Δ8 double bond arising from the Δ8 desaturase [encoded by *SLD1* (At3g61580) and *SLD2* (At2g46210)] [30••,33]. The LCB C-4 hydroxylase *sbh1/sbh2* double mutant, completely lacking trihydroxy LCBs, is severely dwarfed due to defects in both cell elongation and division and displays constitutive upregulation of HR PCD genes [31••]. Sphingolipidome analysis of *sbh1/sbh2* rosettes revealed hyper-accumulation of total sphingolipids to levels twofold to threefold higher than in wild type plants [31••], and the accumulated sphingolipids contained predominantly C16 FAs rather than the more typical C20–C26 VLCFAs. This phenotype suggested that sphingolipids with ceramides containing C16 FAs and dihydroxy LCBs are unable to support normal plant growth, and these sphingolipids or their metabolites are unable to regulate sphingolipid homeostasis [31••]. It also suggested two classes of ceramide synthases with different substrate specificities: one preferring C16 FAs and dihydroxy LCBs and the other VLCFAs and trihydroxy

Figure 2



Simplified sphingolipid biosynthetic pathway highlighting long-chain base (LCB) and fatty acid (FA) modification reactions and the spatial division of the pathway between the endoplasmic reticulum (ER) and Golgi. Enzymes for each step and corresponding genes mentioned in the text are indicated. As shown, LCBs with dihydroxy LCBs are preferentially acylated with C16 FAs (16:0-CoA) by ceramide synthase I (CS I) primarily for glucosylceramide synthesis. Trihydroxy LCBs are preferentially acylated with very long chain fatty acyl (VLCFA)-CoAs by ceramide synthase II (CS II) for glucosylceramide and glycosyl inositolphosphoceramide (GIPC) synthesis. As also indicated, evidence to date is consistent with free LCBs as substrates for the LCB C-4 hydroxylase (C-4 OHase) and ceramides as substrates for LCB Δ4 and Δ8 desaturases (Δ4 DES, Δ8 DES) and fatty acid α-hydroxylase (FA α-OHase). Abbreviations are as follows: SPT, serine palmitoyltransfersase; KSR, 3-ketosphinganine reductase; GCS, glucosylceramide synthase; KCS, 3-ketoacyl-CoA reductase; ELO, fatty acid elongase; KCR, 3-ketoacyl-CoA reductase; HAD, hydroxyacyl-CoA dehydrase; ECR, enoyl-CoA reductase; n-9 DES, n-9 desaturase; LCBK, LCB kinase; SPHK, sphingosine kinase; LCB-P-Pase, LCB phosphate phosphatase; CERK, ceramide kinase; Cer-P-Pase, ceramide phosphate phosphatase; IPCS, inositol phosphoceramide synthase; PI, phosphatidylinositol; DAG, diacylglycerol.

LCBs [31••]. Accordingly, in the *sbh1/sbh2* mutant, dihydroxy LCB flux is proposed to be shunted largely through the first ceramide synthase class. As described below, this has now been confirmed by characterization of ceramide synthase mutants [34••].

In contrast to the LCB C-4 hydroxylase mutant, the Δ8 desaturase *sld1/sld2* double mutant, lacking Δ8 unsaturated LCBs, is indistinguishable from wild type plants under optimal growth conditions [30••]. However, growth phenotypes are revealed upon exposure of plants to extreme growth conditions. For example, the *sld1/sld2* mutant grown at 0 °C develops a chlorotic appearance and senesces prematurely compared to wild type plants [30••]. In addition, transgenic Arabidopsis engineered by expression of a variant Δ8 desaturase to produce increased ratios of *cis*-Δ8 to *trans*-Δ8 LCBs have increased tolerance to aluminum [35]. Collectively, the data suggest that LCB Δ8 desaturation contributes to the structural properties of the plasma membrane to affect tolerance to environmental extremes. The *sld1/sld2* mutant also has a 50% reduction in GlcCers and a proportional increase in GIPCs, suggesting that Δ8 unsaturation contributes to metabolic or physical partitioning of ceramides between

GlcCer synthesis in the ER and GIPC synthesis in the Golgi [30••].

LCB Δ4 unsaturation in Arabidopsis is largely found in floral tissues and pollen, consistent with the expression pattern of the single LCB Δ4 desaturase gene (At4g04930) [32]. This is in stark contrast to other plant species such as tomato and soybean that contain high levels of LCBs with Δ4 unsaturation (most often as d18:2Δ4*E*,8*Z*/*E*) throughout the plant, found almost exclusively in GlcCers [27]. Arabidopsis LCB Δ4 desaturase null mutants have no detectable alterations in floral morphology or pollen viability, but flowers have ~25% less GlcCer, suggesting a role for Δ4 unsaturation in targeting LCBs to GlcCer synthesis [32]. Consistent with this, *Pichia pastoris* LCB Δ4 desaturase knockout mutants lack GlcCers [36•].

Ceramide FA hydroxylation and desaturation

Variation in desaturation, hydroxylation and chain-length is also found in the amide linked FA of ceramides and complex sphingolipids. Through reverse-genetics, Smith *et al.* [37••] identified one member (*ADS2*, At2g31360) of the acyl-CoA desaturase-like gene family that generate 24:1 and 26:1 VLCFAs of Arabidopsis sphingolipids. The *ads2* mutant has significantly reduced levels of 24:1-CoA and 26:1-CoA, suggesting that desaturation occurs in the acyl-CoA pool [37••]. The absence of these monounsaturated VLCFAs from sphingolipids (and selected lipids such as PS and PE) did not perturb growth or development of the plant, so their functional significance remains to be elucidated [37••].

Sphingolipid FA α-hydroxylation is thought to occur on the ceramide, rather than the free acyl chain [36•,38]. The enzyme responsible for this modification was previously identified in yeast as FAH1, a cytochrome b_5-fusion enzyme (similar to the Δ8 desaturase) [39]. Two functional Arabidopsis orthologs *AtFAH1* (At2g34770) and *AtFAH2* (At4g20870) have been identified by complementation of the yeast *fah1* mutant [39]. Interestingly, both Arabidopsis sequences lack the cytochrome b_5 domain [40], and evidence exists for microsomal cytochrome b_5 as the AtFAH1/2 electron donor to AtFAH1/2 [41]. AtFAH1 and AtFAH2 have discrete chain length preferences, with AtFAH1 most active for VLCFA hydroxylation and AtFAH2 preferring C16 FA substrates [25•,40]. Loss of either *AtFAH1* or *AtFAH2* did not alter plant growth and development, though a double mutant showed reduced leaf and root growth [25•]. The *atfah1/atfah2* mutant displayed a 10-fold increase in ceramide levels and increased resistance to biotrophic pathogens, most likely as a result of increased salicylate in these plants [25•].

Ceramide synthesis and regulation of sphingolipid fatty acid composition

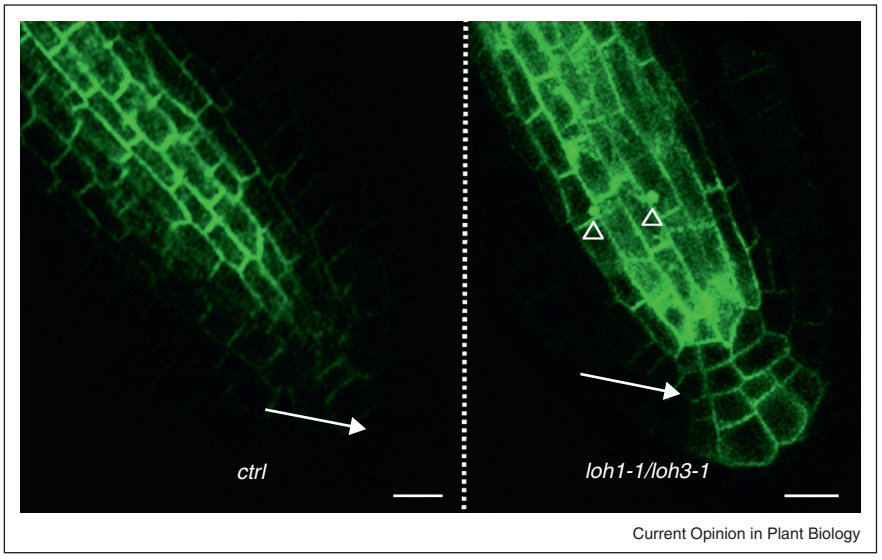
Sphingolipid FA composition is quite distinct from that of other membrane lipids, suggesting that FA chain length plays a critical role in defining sphingolipid function. Plant sphingolipids are highly enriched in VLCFAs with the remainder consisting almost entirely of C16 fatty acids [27]. As discussed above, this is thought to reflect the substrate specificities of the ceramide synthases. Three ceramide synthase genes occur in Arabidopsis: *LOH1* (At3g25540), *LOH2* (At3g19260) and *LOH3* (At1g13580). Knockout of just *LOH2* depleted all sphingolipids having C16 FAs [34••]. No phenotype is associated with this knockout under optimal growth conditions although the plants are extremely sensitive to the sphinganine-analog mycotoxins fumonisin B_1 (FB1) and AAL-toxin that inhibit ceramide synthases [34••]. In contrast, although individual knockouts of *LOH1* or *LOH3* also showed no change in sphingolipid FA profile, the double mutant contained no VLCFA in sphingolipids. The specificities of the *LOH*-encoded ceramide synthases was further confirmed by expression of the proteins in yeast lacking endogenous ceramide synthase [42•].

The *atloh1/atloh3* double knockout is lethal early in seedling development, demonstrating the essential role of sphingolipids containing VLCFA play in plant growth [34••]. Knock-down mutants of *LOH1* and *LOH3* or inhibition of ceramide synthase activity using FB1 led to aberrant trafficking of the plasma membrane proteins AUX1 and PIN1 and their accumulation in intracellular bodies (Figure 3), indicating that VLCFA-containing sphingolipids play a critical role in protein trafficking that cannot be compensated for by FAs with a shorter chain length [34••]. Consistent with this, FA elongation mutants with reduced sphingolipid VLCFA also display defective endomembrane trafficking [43–45]. It remains to be determined whether the VLCFA-containing sphingolipids influence membrane physical properties that impact trafficking or whether they are required for optimal function of specific trafficking-associated proteins.

Sphingolipid structure and signaling

Sphingolipids play important roles in signaling pathways and in some instances specific sphingolipids have been identified as components of pathways, and potential downstream targets have been uncovered. Evidence that ceramides promote HR and associated PCD comes from Arabidopsis *acd5* mutants deficient in ceramide kinase activity that exhibit ceramide accumulation, enhanced susceptibility to infection, and precocious apoptotic-like cell death [8]. In Arabidopsis lines carrying the resistance gene *RPW8*, knockout of *ERH1* (At2g37940), one of the three IPC synthase genes, results in enhanced HR-like cell death that is preceded by ceramide accumulation in the mutant, with 16:0-containing ceramides exhibiting the most profound increase [10•]. It has been suggested that SA-dependent resistance exhibited by *acd5*, *erh1* and *atfah1/atfah2* mutants is directly linked to the over-accumulation of ceramides. However, unlike *acd5* and

Figure 3



Mislocalization of PIN1-GFP in ceramide synthase mutants of Arabidopsis with reduced sphingolipid-VLCFA content (reproduced from [34••] copyright American Society of Plant Biologists). In primary roots of wild-type plants (ctrl), PIN1::PIN1-GFP fluorescence (green) is seen only in cells of the vascular cylinder and is localized to the plasma membrane. No expression is seen in the columella cells (arrow). In loh1/3 roots with reduced sphingolipid-VLCFA content, PIN1::PIN1-GFP fluorescence is found in the columella cells (arrow) and PIN1-GFP accumulates in aggregates within the cells of the cortex (arrow heads).

erh1, the hydroxylase-deficient mutant does not display a PCD-like phenotype, leading Konig *et al.* [25•] to propose that the relationship between ceramides, SA and PCD may not be causal and that other factors (such as elevated free LCBs) may play an important role. While differences in the accumulation of selected ceramide species may account for this difference, PCD also appears to be promoted by elevated LCBs, particularly d18:0. The LCB d18:0 accumulated in both FB1-treated and paraquat-treated plants, leading to PCD in both cases. The mutant, *fbr11-1*, with reduced expression of *LCB1*, exhibits resistance to FB1 [9]. Similarly, *lcb2a* mutants avoid FB1-induced d18:0 accumulation and PCD, and it has been reported that the mitogen activated protein kinase MPK6 is a downstream component of this d18:0-mediated PCD pathway [46•].

LCB-Ps have been implicated in guard cell signaling in response to ABA. Following an initial study implicating d18:1-P in stomatal closure in *Commelina communis* [13], subsequent studies in Arabidopsis showed that LCB kinase activity is transiently stimulated by ABA and that the sole G-protein α-subunit is a downstream component of LCB-P in the ABA signaling pathway [12]. Recently, phospholipase D (PLD) was identified as a component of the ABA pathway downstream of t18:0-P [47•]. However, phosphatidic acid (PA) produced by PLD has been shown to activate LCB kinase activity, and so a cooperative synergism between LCB kinase/LCB-P and PLD/PA in the ABA signaling pathway is likely [47•]. That exogenous t18:0-P can influence guard cell behavior [48] and mutants lacking the LCB Δ4 desaturase exhibit normal stomatal behavior in response to ABA [32] point to t18:0-P as the likely signal molecule in Arabidopsis. ABA is known to influence other plant processes such as germination, and evidence suggests that those associated pathways also involve LCB-P [14].

Rapid transient formation of t18:0-P in response to low temperature has been reported [18•,20]. Only mutants lacking one particular LCB kinase gene, *LCBK2* (At2g46090), failed to exhibit low temperature-induced t18:0-P synthesis [18•]. MPK6 activity was stimulated in wild type but not in *lcbk2* mutants upon shifting temperature, and the activity of MPK6 in cultured cells was stimulated by exogenous t18:0-P, but not by d18:0-P or t18:0, suggesting that t18:0-P regulates (directly or indirectly) MPK6 [18•]. The reports by Dutilleul *et al.* [18•] and Saucedo-Garcia *et al.* [46•] implicate MPK6 as a downstream transducer for distinct sphingolipids, responding to environmental cues (low temperature) in the presence of elevated t18:0-P but promoting PCD in the presence of elevated d18:0. Suggestive of some commonalities with ABA signaling, a leading candidate for the upstream signal stimulating LCBK2 enzyme activity in response to low temperature is PA, given that it stimulates the activity of LCB kinase [47•] and is generated rapidly by PLD in response to a temperature shift [49].

Sphingolipid structure and homeostasis regulation

Despite compelling evidence that the various species of sphingolipids have distinct functions, very little is known about the regulation of their synthesis. Mediation of homeostasis is critical for maintaining sufficient amounts of sphingolipids to support growth, while limiting accumulation of PCD-inducing species. As the committed enzyme of sphingolipid synthesis, SPT has been considered a likely target for regulation and recent developments have set the stage for unraveling the control of SPT activity in plants. In particular, yeast LCB1/LCB2 heterodimers that make up the core of SPT are organized in a complex that includes the small activating Tsc3p protein and the inhibitory ORM and Sac1p proteins [50••,51••]. Moreover, the ORMs have been shown to be downstream effectors of a Torc2/Pkh/Ypk kinase cascade that couples sphingolipid availability to SPT activity. In the current model, sphingolipid depletion results in Ypk1/2-mediated phosphorylation of the N-termini of the ORMs, thereby relieving inhibition of SPT [52,53,54]. Two homologs of the recently identified human ssSPTs (small activating subunits of SPT) occur in Arabidopsis that are functional orthologs of yeast Tsc3p [55••] and there are also two ORM homologs in Arabidopsis. Like the mammalian ORM homologs, which have been implicated in regulation of sphingolipid homeostasis [50••,56], the Arabidopsis ORMs lack the N-terminal extension whose phosphorylation status controls ORM inhibition of yeast SPT and thus it appears that the higher eukaryotic ORMs are regulated differently from the yeast ORMs. Studies of yeast mutants lacking various sphingolipid modifying enzymes point to the importance of ceramide in ORM regulation [50••,51••,52], but it is not yet clear whether specific sphingolipids are directly sensed or whether, for example, altered membrane biophysical properties activate the pathway. The observation that the *sbh1/sbh2* mutant has reduced levels of the trihydroxy LCB-VLCFA ceramides and twofold to threefold higher sphingolipid levels relative to wild-type [31••] raises the possibility that a trihydroxy-containing sphingolipid or biosynthetic intermediate controls SPT activity via the ORMs in plants, which is likely to be an important area for future research.

Conclusions

Through the combination of sphingolipidomics and Arabidopsis mutant characterization, the functional significance of specific sphingolipid LCB and FA structural modifications has emerged. It is now apparent that the lack of a single hydroxyl group or double bond can severely impact flux, which in turn has provided important clues about underlying sphingolipid biosynthetic reactions. For example, the study of defective LCB C-4 hydroxylation and the associated aberrant accumulation of C16 FA-containing sphingolipids has provided insights into the role that ceramide synthases play in dictating FA chain-length of sphingolipids as well as possible clues about the regulation of sphingolipid homeostasis [31••]. Links between sphingolipid structural modifications and endomembrane trafficking [34••,43–45], PCD induction [46•], temperature responses [18•,19,20], and ABA-dependent guard cell signaling [12,13,48] have also been shown. It is likely that additional insights into the significance of sphingolipid structure will be obtained from studies of plants beyond Arabidopsis that have even greater sphingolipid diversity. For example, LCB Δ4 unsaturation, which is nearly absent in most organs of Arabidopsis [32], is one of the major structural modifications in GlcCers of crop species including tomato and soybean [21]. Also largely unexplored is the functional significance of the polar head groups of complex sphingolipids. What is the individual and relative importance of GlcCers and GIPCs? What is the functional basis for differences in the composition and extent of glycosylation of GIPCs? Answers to questions such as these, together with information obtained to date, may provide a path for tailoring sphingolipid composition to enhance the productivity and abiotic and biotic stress tolerance of crop plants.

Acknowledgements

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