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Review

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Plant sphingolipids: decoding the enigma of the Sphinx

Author for correspondence:
Carl K-Y. Ng
Tel: +353 1 716 2250
Email: carl.ng@ucd.ie

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Mickael O. Pata[1], Yusuf A. Hannun[2] and Carl K-Y. Ng[3]

[1]Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR 441-2594 (INRA-CNRS), Chemin de Borde Rouge BP 52627, 31326 Castanet-Tolosan, France; [2]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave, Charleston, SC 29425, USA; [3]School of Biology and Environmental Science, University College Dublin, Belfield, Dublin 4, Ireland

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Summary

Sphingolipids are a ubiquitous class of lipids present in a variety of organisms including eukaryotes and bacteria. In the last two decades, research has focused on characterizing the individual species of this complex family of lipids, which has led to a new field of research called 'sphingolipidomics'. There are at least 500 (and perhaps thousands of) different molecular species of sphingolipids in cells, and in Arabidopsis alone it has been reported that there are at least 168 different sphingolipids. Plant sphingolipids can be divided into four classes: glycosyl inositol phosphoceramides (GIPCs), glycosylceramides, ceramides, and free long-chain bases (LCBs). Numerous enzymes involved in plant sphingolipid metabolism have now been cloned and characterized, and, in general, there is broad conservation in the way in which sphingolipids are metabolized in animals, yeast and plants. Here, we review the diversity of sphingolipids reported in the literature, some of the recent advances in our understanding of sphingolipid metabolism in plants, and the physiological roles that sphingolipids and sphingolipid metabolites play in plant physiology.

I. Introduction

Sphingolipids are a ubiquitous class of lipids present in a variety of organisms including eukaryotes and bacteria (reviewed in Sperling & Heinz, 2003; Lynch & Dunn, 2004; Merrill *et al.*, 2007, 2009; Spiegel & Milstien, 2003; Hannun & Obeid, 2008; Pruett *et al.*, 2008). In the last two decades, research has focused on characterizing the individual species of this complex family of lipids, which has led to a new field of research called 'sphingolipidomics' (Merrill *et al.*, 2005, 2007; Bielawski *et al.*, 2006; Sullards *et al.*, 2007; Pruett *et al.*, 2008). Because of the complexity of sphingolipids, and their diversity among plants and also among organs within the same plant, powerful analytical

tools are required to directly identify individual molecules. The techniques used include capillary gas chromatography, infrared spectrometry, high-performance liquid chromatography and mass spectrometry (Cahoon & Lynch, 1991; Sullards *et al.*, 2000; Ng *et al.*, 2001; Ternes *et al.*, 2002; Bielawski *et al.*, 2006; Markham *et al.*, 2006; Wang *et al.*, 2006; Markham & Jaworski, 2007; Shi *et al.*, 2007). It is noteworthy that the success of liquid chromatography-tandem mass spectrometry (LC/MS/MS) approaches for characterizing sphingolipids is dependent on the development of efficient extraction protocols because of the diverse polarity associated with sphingolipids (Markham *et al.*, 2006; Merrill *et al.*, 2007, 2009).

The total content of sphingolipid species within a given plant or organ/tissue is given the term 'sphingolipidome' (Spassieva & Hille, 2003; Pruett *et al.*, 2008; Merrill *et al.*, 2009). Although there is great diversity in the composition of the sphingolipidome from different species, the basic building block of sphingolipids is an amino alcohol long-chain base (LCB), which is composed predominantly of 18 carbon atoms. The LCB is characterized by the presence of a hydroxyl group at C1 and C3 and an amine group at C2 (2-amino-1,3-dihydroxyalkane; Fig. 1).

To form a ceramide, the amine group of the LCB is acylated with a fatty acid (FA) generally composed of 14–26 carbon atoms in plant cells (Sperling & Heinz, 2003; Lynch & Dunn, 2004). This *N*-acyl-LCB is the backbone of the sphingolipids detected in cells, and therefore the basic building block for the synthesis of more complex sphingolipids. The basic ceramide structure can be further modified through changes in chain length, methylation, hydroxylation and/or degree of desaturation of both the LCB and FA moieties (Fig. 2). Further modifications involve the conjugation of the primary hydroxyl (OH) group of the LCB moiety (Fig. 2), resulting in a polar headgroup which can be a phosphoryl group (ceramide phosphates), mono- or pluri-hexose (glycosylceramides), and an inositol phosphate group (IP) (Fig. 2) (Spassieva & Hille, 2003; Sperling & Heinz, 2003; Lynch & Dunn, 2004).

There are at least 500 (and perhaps thousands of) different molecular species of sphingolipids in cells (Vesper *et al.*,

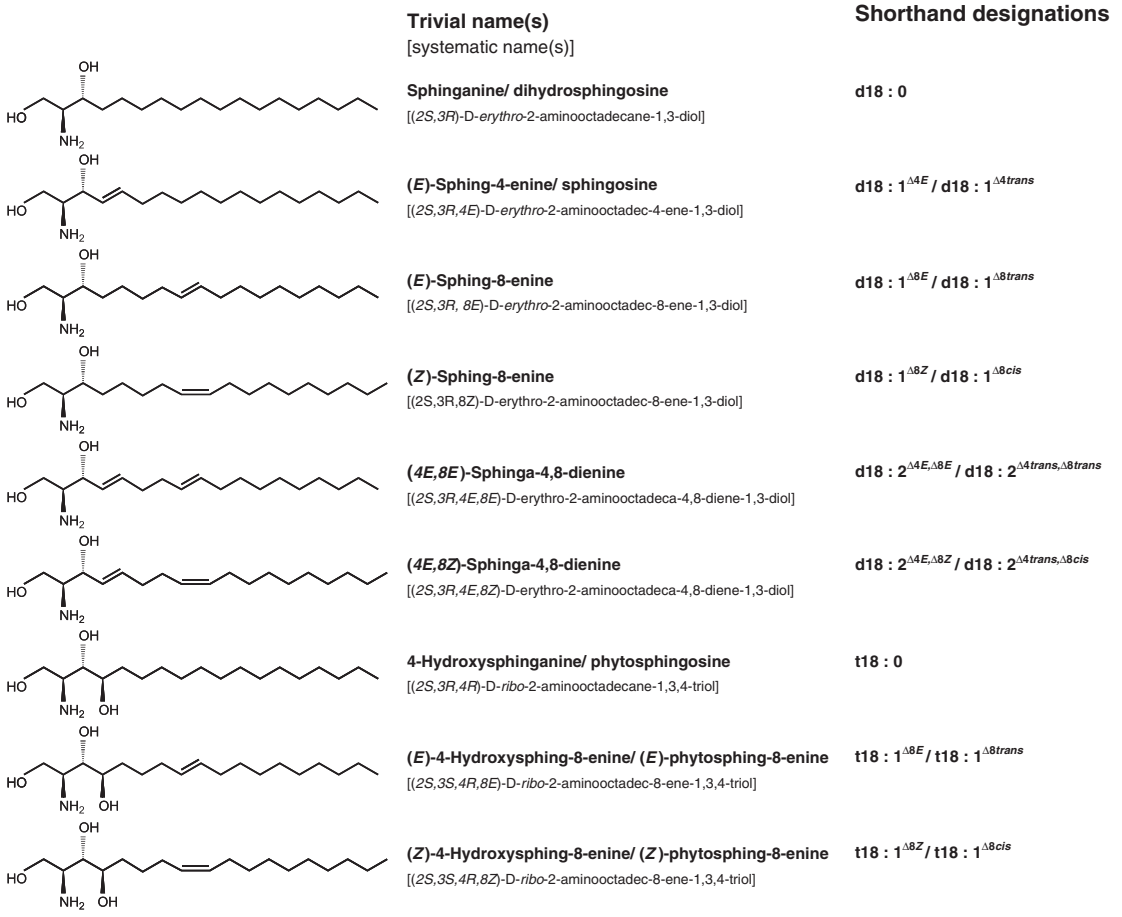

Fig. 1 Structures of representative C18 long-chain bases (LCBs) found in plants. Trivial names and systematic names are consistent with IUPAC (International Union of Pure and Applied Chemistry) (http://www.chem.qmul.ac.uk/iupac/lipid/) regulations and shorthand designations are given for each LCB. All dihydroxy (d) and trihydroxy (t) LCBs are naturally occurring in plants with D-*erythro* and D-*ribo* configurations, respectively. These LCBs are detected as parts of ceramides or complex sphingolipids or as free LCBs.

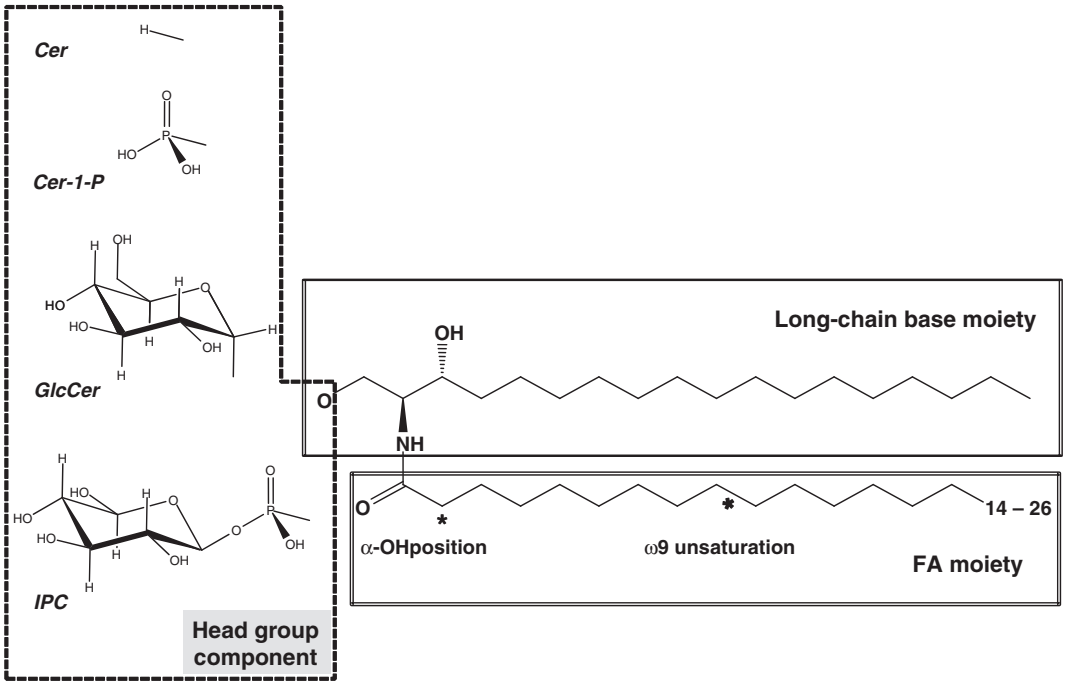

Fig. 2 Schematic representation of complex sphingolipids from plants. The general structure of complex sphingolipids is based on a hydrophobic ceramide core and a hydrophilic head group. The ceramide core is made up of two moieties, a long-chain base (LCB) and a fatty acid (FA) linked via an amide bond. The LCB moiety can vary, and some of the common LCBs are shown in Fig. 1. The FA can vary in length, saturation and hydroxylation. The ceramide core shown here is dihydroceramide, which is a biosynthetic precursor of ceramide cores in the *de novo* pathway. Cer, ceramide; GlcCER, glycosylceramide; IPC, inositolphospho ceramide. IPC can be further glycosylated with different sugar residues.

1999; Hannun & Luberto, 2000; Futerman & Hannun, 2004). In Arabidopsis alone, it has been reported that there are at least 168 different sphingolipids (Markham & Jaworski, 2007) and 30 different ceramide cores have been identified in rye (*Secale cereale*) leaves (Cahoon & Lynch, 1991). It is therefore no surprise that the potential exists, in any organism, for the generation of highly diverse and complex sphingolipids through variations in the composition of the FA, LCB and headgroup modifications. Plant sphingolipids can be divided into glycosyl inositol phosphoceramides (GIPCs), glycosylceramides, ceramides, and free LCBs. The structures of GIPCs, glycosylceramides, ceramides, and LCBs are illustrated in Figs 1 and 2, and Tables 1–6. Numerous enzymes involved in plant sphingolipid metabolism have now been cloned and characterized. In general, there is broad conservation in the way in which sphingolipids are metabolized in animals, yeast and plants. Sphingolipids can be formed via two pathways: the *de novo* pathway, starting with the condensation of a serine with an acyl-CoA; and the salvage pathway, where ceramides and LCBs are released from more complex sphingolipids, followed by channeling of the metabolites formed into the synthetic pathway (Merrill, 2002; Hannun & Obeid, 2008; Kitatani *et al.*, 2008) (Figs 3,4). In the following sections, we shall review the functions of the major plant sphingolipid classes. For in-depth reviews of plant sphingolipid metabolism, readers are referred to excellent reviews by Spassieva & Hille (2003), Sperling & Heinz (2003), and Lynch & Dunn (2004).

II. Ceramide metabolism

Ceramide can be formed via two pathways: the FA-CoA-dependent and free FA-dependent pathways. The FA-CoA-dependent pathway is the major route through which ceramide is synthesized in plants (reviewed by Sperling & Heinz, 2003; Lynch & Dunn, 2004). The formation of the ceramide core (comprising the LCB and the FA moieties) occurs via the condensation of an FA with the amino group of sphinganine (dihydrosphingosine, d18:0). This reaction is catalyzed by dihydroceramide synthase (DHCS; E.C. 2.3.1.24) and the reaction is FA-CoA dependent. Plant DHCSs have been shown to utilize a range of FA-CoAs (C16 to C24) but not α-OH FA-CoAs as substrates (reviewed by Sperling & Heinz, 2003; Lynch & Dunn, 2004). However, the major forms of ceramide, glycosylceramide and GIPC contain α-OH FAs, suggesting that FA hydroxylation is likely to be downstream of ceramide formation. Ceramides can also be formed from the *N*-acylation of the amino group of an LCB with a free FA acting as the acyl donor (Merrill, 2002; Spassieva & Hille, 2003; Sperling & Heinz, 2003; Lynch & Dunn, 2004). This reaction is catalyzed by the reverse activity of ceramidases.

Table 1 Main characteristics of plant glycosyl inositol phosphoceramides (GIPCs)

Family	Species	Tissue	Core headgroup	Additional sugars	LCB profile (mol%)	FA composition (mol%)	References
Linaceae	*Linum usitatissimum*	Seed	PGL CPPS	Ara, Gal, Man, Fuc	70% t18:1$^{\Delta 8}$ 17% t18:0	44% αOH C24 17% αOH C25 11% αOH C26	a-d
Poaceae	*Zea mays*	Seed	PGL CPPS	Ara, Gal	t18:0	65% (αOH C24; αOH C26) 24% (C16; C18) 11% C24	a-c
	Triticum aestivum	Seed	(Inositol-P, GlcN, Ara, Gal, Man)		t18:0		a
Fabaceae	*Glycine max*	Seed	(Inositol-P, GlcN, Ara, Gal, Man) CPPS	 Ara, Gal, Fuc, Man	t18:0 t18:1$^{\Delta 8}$	95% (C16; C18) 5% αOH C24	a, c
	Arachis hypogaea	Seed	(Inositol-P, GlcN, Ara, Gal, Man)				a
	Phaseolus vulgaris	Leaf	Hexosamine-hexuronic acid-inositol-P	Ara, Gal, Man	53% t18:1$^{\Delta 8}$ 32% t18:0	48% αOH C24 20% αOH C22 10% αOH C26	d
Malvaceae	*Gossypium* spp.	Seed	(Inositol-P, GlcN, Ara,Gal, Man)		t18:0		a
Asteraceae	*Helianthus annus*	Seed	(Inositol-P, GlcN, Ara, Gal, Man)		t18:0		a
	Carthamus tinctorius	Seed	CPPS	Ara, Gal, Fuc, Man			c
Solanaceae	*Nicotiana tabacum*	Leaf	PSL-I	[Ara_2Gal_2]; [Ara_3Gal_2]; [Ara_4Gal_2]		52–58% αOH C24 10–18% αOH C25 11–13% αOH C26 10–12% αOH C22	e-h
			PSL-II	[Ara_3Gal]; [$Ara_{2or3}Gal_2$]; [Ara_2Gal_2Man]		50–55% αOH C24 11–17% αOH C22 11–13% αOH C26 10–13% αOH C25	e, f
			GPC	Ara, Gal			h
Brassicaceae	*Arabidopsis thaliana*	Leaf			73% t18:1$^{\Delta 8}$ 15% t18:0	αOH C24:1 > αOH C24 > αOH C20 > αOH C26 = αOH C16	i, j

The names of the core headgroups follow the defunct names given to the GIPCs and are detailed in Table 2. The early characterization in seeds did not establish a model for the structure of the headgroup but only its rough composition, which is indicated in brackets. Additional sugars of 13 or more have been determined in tobacco leaves (Kaul & Lester, 1978).
Ara, arabinose; Fuc, fucose; Gal, galactose; Man, mannose; CPPS, ceramide phosphate polysaccharide; GPC, glycophosphoceramide; PGL, phytoglycolipid; PSL, phosphosphingolipid.
References: a, b, Carter *et al.* (1958, 1964)); c, Carter & Kisic (1969); d, Carter & Koob (1969); e, f, Kaul & Lester (1975, 1978)); g, h, Hsieh *et al.* (1978, 1981)); i, Markham *et al.* (2006); j, Markham & Jaworski (2007).

Table 2 Structure of the core headgroups in glycosyl inositol phosphoceramides (GIPCs)

Headgroup name	Headgroup structure	References
PGL	Glucosamine-hexuronic acid-inositol-P |? Man	a, b
CPPS	Hexuronic acid-inositol-P	c
PSL-I	N-acetylglucosamine (α1 → 4)-glucuronic acid (α1 → 2)-*myo*-inositol-1-*O*-P	e-h
PSL-II	Glucosamine-glucuronic acid-inositol-P	e, f
GPC	N-acetylglucosamine (α1 → 4)-glucuronic acid (α1 → 2)-*myo*-inositol-1-*O*-P | (? ← 1α) Man	h

Two different classes of GIPCs were characterized by Carter and co-workers: phytoglycolipid (PGL) and ceramide phosphate polysaccharide (CPPS). The latter is devoid of hexosamine. The link position of the mannose (Man) group was not determined with precision but later appeared to be on the inositol group of a glycophosphoceramide (GPC) core co-purified with a Gal(α → 4)-phosphosphingolipid (PSL)-I core (Hsieh *et al.*, 1981).
References: a, b, Carter *et al.* (1958, 1964)); c, Carter & Kisic (1969); e, f, Kaul & Lester (1975, 1978)); g, h, Hsieh *et al.* (1978, 1981)).

Once the dihydroceramide (d18:0-ceramide) is formed, it can be further modified by C4-hydroxylation of the LCB moiety to yield phytoceramide (t18:0-ceramide), although this reaction can also occur through the use of sphinganine (d18:0) as the substrate in the endoplasmic reticulum (ER) (Wright *et al.*, 2003; Chen *et al.*, 2008). The ceramides can then be channeled for further modifications (Fig. 3), resulting in the formation of more complex sphingolipids such as GIPCs and glycosylceramides (Fig. 3). They can also be used as substrates for the synthesis of ceramide phosphates (Liang *et al.*, 2003; Fig. 4). Ceramides can also be broken down by the activity of ceramidases, leading to the formation of free LCBs.

Plant glycosylceramide synthases have been characterized using different microsomal preparations (Nakayama *et al.*, 1995; Lynch *et al.*, 1997; Cantatore *et al.*, 2000) and an ortholog from cotton (*Gossypium arboreum*) has been cloned (Leipelt *et al.*, 2001). It is generally accepted that glycosylceramides are synthesized in the ER and/or plasma membrane whereas GIPCs are synthesized in the Golgi (Bromley *et al.*, 2003; Hillig *et al.*, 2003; Wang *et al.*, 2008). The initial committed step of GIPC synthesis is the formation of IPC, which requires the transfer of inositol phosphate from the phospholipid phosphatidylinositol (PI) to ceramide. This reaction results in the release of diacylglycerol by-products and is catalyzed by an IPC synthase.

IPC synthase activity has been characterized in wax bean (*Phaseolus vulgaris*) microsomes (Bromley *et al.*, 2003) and a variety of other plants, but particularly high activities have been detected in the Fabaceae (Bromley *et al.*, 2003). IPC synthase is able to catalyse IPC synthesis using nonhydroxy and hydroxyceramide as substrates. However, the wax bean IPC synthase exhibits greater activity towards ceramides with α-OH FA (Bromley *et al.*, 2003). IPC synthase has been shown to be an important regulator of the plant ceramide pool. In the Arabidopsis IPC synthase mutant *erh1* (enhancing RPW8-mediated HR-like cell death 1), the concentration of ceramides is dramatically increased and the plants exhibit enhanced hypersensitive response (HR)-like cell death when challenged with powdery mildew (Wang *et al.*, 2008).

To date, α-hydroxylation of the FA moiety of plant sphingolipids has not been characterized *in vitro*, although ceramide synthase has been shown to be inhibited by α-OH FA, and it is therefore generally believed that α-hydroxylation occurs after ceramide core formation (Sperling & Heinz, 2003; Warnecke & Heinz, 2003; Lynch & Dunn, 2004). The α-hydroxylase activity in the protozoan *Tetrahymena pyriformis* has been demonstrated to exhibit preference for FA from ceramide or complex sphingolipids (Kaya *et al.*, 1984). The yeast fatty acid 2-hydroxylase gene (FAH1/SCS7) was shown by deletion/disruption to be involved in the α-hydroxylation of C26 very long chain fatty acid (VLCFA) (reviewed by Sperling & Heinz, 2003; Lynch & Dunn, 2004). Two putative α-hydroxylase (AtFAH) homologs have been identified in Arabidopsis, both of which were able to restore α-hydroxylase activity in the *fah1Δ* yeast mutant strain (Nagano *et al.*, 2009). Interestingly, the Arabidopsis α-hydroxylases lack the cytochrome b_5 (cyt b5) domain, unlike their yeast counterparts (reviewed by Sperling & Heinz, 2003; Lynch & Dunn, 2004). Nagano *et al.* (2009) showed that the AtFAHs can interact with Arabidopsis cytochrome b_5 (AtCb5) and that the AtFAH–AtCb5 complex is subjected to regulation by the Arabidopsis Bax inhibitor-1 (AtBl-1) protein, leading the authors to suggest that AtBl-1, through its interaction with the AtCb5–AtFAH complex, can regulate cell death via changes in the concentrations of α-OH ceramides with VLCFAs (Nagano *et al.*, 2009).

Table 3 Main characteristics of the fatty acid (FA) moiety of plant glycosylceramides

Family	Species	Tissue	Non-OH FA	Short αOH FA (C < 20)	Saturated αOH VLCFA	Unsaturated αOH VLCFA	References
Poaceae	*Oryza sativa*	Seed bran[1]	11	5.6–8	83.3–91		a, b
		Endosperm[1]	9	5.4	86		a
		Leafy stem	< 1	2.1	97.9		c
		Leaf		1.5	94		d
	Zea mays	Leaf		8.4	86.5		d
		Root	19.1	12.6	68.3		e
	Avena sativa	Leaf		6.8–7	12.1–77.5	10.8–80.5	d, f
		Leaf (CA)		5.8	6.9	87	f
		Root				> 90	g
	Triticum aestivum	Flour	2	71.7	26		h
		Hypocotyl		53	44		b
		Leaf		8.8	58.8	27.4	d
	Secale cereale (winter rye cv. Puma)	Leaf		3–18.8	25–37.3	42.5–61	d, f, i
		Leaf (CA)		11.3	24.3	64.3	f
Brassicaceae	*Arabidopsis thaliana*	Leaf		82	4.6	5.9	j
		Leaf (CA)		85.2	5.8	6.5	j
	Brassica oleracea (cabbage)	Leaf		32	68		d
	Brassica oleracea (broccoli)	Leaf		29	57	14	d
Fabaceae	*Glycine max*	Seed		> 95			k
	Pisum sativum	Leaf		55	44		d
		Seed[1]	2	59.6	33.3		l
	Phaseolus vulgaris	Leaf	7	1	77		m
Solanaceae	*Solanum tuberosum*	Leaf		82	17		d
		Tuber	6.4–20.9	78–88.5	1.7–4.4	0.2–1.5	n
	Capsicum annuum	Leaf		86	13		d
		Red-ripe fruit		60	40		o
	Lycopersicon esculentum	Leaf		78	23		d
		Red-ripe fruit		70	30		o
Vitaceae	*Vitis vinifera*	Leaf		55.3	39.7		p
	V. vinifera cv. Zweigeltrebe[2]	Leaf		48	49.5		p
Convolvulaceae	*Ipomoea batatas*	Tuber	10.6	80	10.8	1	n
		Leaf		8	88		d
Cucurbitaceae	*Cucurbita maxima*	Leaf		74	26		d
	Cucumis sativus	Leaf		62	38		d

The data reported here are expressed in mol% of total glycosylceramide.
[1]Only the composition of monoglucosylceramide has been considered.
[2]This cultivar is considered freezing-sensitive compared with the wild-type species.
CA, cold acclimated (4 wk); VLCFA, very long chain fatty acid.
References: a, Fujino *et al.* (1985); b, Takakuwa *et al.* (2005); c, Ohnishi *et al.* (1985); d, Imai *et al.* (1995); e, Bohn *et al.* (2001); f, Uemura & Steponkus (1994); g, Norberg *et al.* (1996); h, Laine & Renkonen (1974); i, Lynch *et al.* (1992); j, Uemura *et al.* (1995); k, Sullards *et al.* (2000); l, Ito *et al.* (1985); m, Carter & Koob (1969); n, Bartke *et al.* (2006); o, Whitaker (1996); p, Kawaguchi *et al.* (2000).

Table 4 Main characteristics of the long-chain base (LCB) moiety of plant glycosylceramides

Family	Species	Tissue	d18	d18:1$^{\Delta 4E}$	d18:1$^{\Delta 8E}$	d18:1$^{\Delta 8Z}$	d18:2$^{\Delta 4E\Delta 8E}$	d18:2$^{\Delta 4E\Delta 8Z}$	t18:0	t18:1$^{\Delta 8E}$	t18:1$^{\Delta 8Z}$	References
Poaceae	*Oryza sativa*	Seed bran[1]	0.3–1	1–2.5	1–1.8[3]		13–16.5	50–53.3	3.3–6	3–6.1	16.2–25	a, b
		Endosperm[1]	1	5.9	2.2[3]		34.6	40.4	1.2	2.8	11.9	a
		Leafy stem	< 0.1	1.4	0.4[3]		16.3	22.6	2.7	56.6[3]		c
		Leaf	0.1		0.3	0.3	11.5	34.3	0.8	3.1	49.6	d
	Zea mays	Leaf	< 0.1		0.2	1	17.3	55.7	0.4	1.6	23.8	d
	Avena sativa	Leaf	< 0.1		1.6	0.7	13.5	16.2	1.6	14.8	51.6	d
		Leaf					35.9[3]			63.1[3]		e
		Leaf (CA)					31.6[3]			67.9[3]		e
		Root					100[3]					f
	Triticum aestivum	Hypocotyl	14	1	25	47	3	5	2	1	2	g
		Leaf	0.2		1.3	3.2	5.2	9.4	0.9	6.9	72.9	d
	Secale cereale (winter rye cv. Puma)	Leaf					3	14		6	70	h
		Leaf			1.1[3]		21.8[3]			69.2[3]		e
		Leaf (CA)			1.1[3]		17.1[3]			66.6[3]		e
Brassicaceae	*Arabidopsis thaliana*	Leaf	0.4–1.6		22.7–27.9	2–4.8			1.1	21.5–32.9	40.9–44.2	i, j
	Brassica oleracea	Inflorescence					18.6	3		22.4	56	j
Fabaceae	*Glycine max*	Seed								> 95[3]		k
		Leaf	0.3		3	1.3	33.5	14.2	0.7	22.2	24.8	d
	Pisum sativum	Seed[1]	1.8	0.4	51.1[3]		9.6	0.6	0.8	35.7[3]		l
		Leaf	0.7		17.7–29.3	11.5–35.6	4.8	2.3	0.8–1.1	5.6–20.8	28.4–41.4	d, j
		Root			30.6	14.3	9.5	2.2		21.8	21.6	j
	Phaseolus vulgaris	Leaf	4						7	52[3]		m
Solanaceae	*Solanum tuberosum*	Tuber			4.9[3]		91.4[3]			3.7[3]		n
	Capsicum annuum	Leaf	1.1		21.3	29	24.2	13.4	0.2	4.7	6.1	d
		Red-ripe fruit			17[3]		45[3]			34[3]		o
	Lycopersicon esculentum	Leaf	0.1		0.6	0.1	57.8	20	0.3	16.9	4.2	d
		Red-ripe fruit					76[3]			27[3]		o
	Nicotiana tabacum	Leaf					22.1	72.3		2.2	3.4	j
Vitaceae	*Vitis vinifera*	Leaf	< 0.1		0.6	0.8	18.5	55.7	0.2	5.7	18.5	p
	V. vinifera cv. Zweigeltreb[2]	Leaf	0.1		1.2	1.1	26.7	50.4	0.3	8.3	11.9	p
Convolvulaceae	*Ipomoea batatas*	Leaf	0.2		1.1	0.3	14.7	17.7	0.6	12.4	53	d
		Tuber			4.4[3]		86.1[3]			9.5[3]		n
Cucurbitaceae	*Cucurbita maxima*	Leaf					54	17		19	10	d
	Cucumis sativus	Leaf					67	2		24	7	d
Amaranthaceae	*Spinacia oleracea*	Leaf	0.1		2.9–5.1	0.9	44.6–65	1.4–5.5	0.1–19.7	7.4–10.4	16.9–21	d, j

The data reported here are expressed in % of total glycosylceramide.
[1]Only proportion for monoglucosylceramide has been considered.
[2]This cultivar is considered freezing-sensitive compared with the wild-type species.
[3]Value independent of the isomer configuration.
CA, cold acclimated (4 wk).
References: a, Fujino *et al.* (1985); b, Takakuwa *et al.* (2005); c, Ohnishi *et al.* (1985); d, Imai *et al.* (1997); e, Uemura & Steponkus (1994); f, Norberg *et al.* (1996); g, Takakuwa *et al.* (2005); h, Lynch *et al.* (1992); i, Markham *et al.* (2006); j, Sperling *et al.* (2005); k, Sullards *et al.* (2000); l, Ito *et al.* (1985); m, Carter & Koob (1969); n, Bartke *et al.* (2006); o, Whitaker (1996); p, Kawaguchi *et al.* (2000).

Table 5 Main characteristics of the fatty acid (FA) moiety of plant ceramides

Family	Species	Tissue	Non-OH FA	Short αOH FA (C < 20)	Saturated αOH VLCFA	Unsaturated αOH VLCFA	References
Poaceae	*Oryza sativa*	Seed bran*	28		70		a
		Endosperm*	32	1.9	66.1		a
		Leafy stem	30	< 0.2	66.9		b
Fabaceae	*Pisum sativum*	Seed*	7	18.6	71.4		c
	Phaseolus vulgaris	Leaf			100		d
Solanaceae	*Solanum tuberosum*	Tuber	9.5–71.5	34.5–53.5	16.5–31	0.5–8	e
Convolvulaceae	*Ipomoea batatas*	Tuber	37	35	29		e

The data reported here are expressed in mol% of total ceramide.
VLCFA, very long chain fatty acid.
References: a, Fujino *et al.* (1985); b, Ohnishi *et al.* (1985); c, Ito *et al.* (1985); d, Carter & Koob (1969); e, Bartke *et al.* (2006).

Table 6 Main characteristics of the long-chain base (LCB) moiety of plant ceramides

Family	Species	Tissue	d18	d18:1$^{\Delta 4E}$	d18:1$^{\Delta 8E}$	d18:1$^{\Delta 8Z}$	d18:2$^{\Delta 4E\Delta 8E}$	d18:2$^{\Delta 4E\Delta 8Z}$	t18:0	t18:1$^{\Delta 8E}$	t18:1$^{\Delta 8Z}$	References
Poaceae	*Oryza sativa*	Seed bran	2.2	0.5		7.4	1.6	2.6	68.8		16.9	a
		Endosperm	10.1	5.8		< 0.1	4.7	24.4	45.8		9.2	a
		Leafy stem	1.8	< 0.1		4.3	0.2	1.5	83		9.2	b
Brassicaceae	*Arabidopsis thaliana*	Leaf		1.1					15.9–47.7	49.3–76.3	2.9–6.7	f, g
Fabaceae	*Pisum sativum*	Seed	3.1					1.6	35.1		60.2	c
	Phaseolus vulgaris	Leaf							24	54[1]		d
Solanaceae	*Solanum tuberosum*	Tuber			7.4[1]		57.5[1]			35.1[1]		e
Convolvulaceae	*Ipomoea batatas*	Tuber§			10.3[1]		37.9[1]			51.7[1]		e

The data reported here are expressed in mol% of total ceramide, except § (wt%).
[1]Value independent of the isomer configuration.
References: a, Fujino *et al.* (1985); b, Ohnishi *et al.* (1985); c, Ito *et al.* (1985); d, Carter & Koob (1969); e, Bartke *et al.* (2006); f, Sperling *et al.* (2005); g, Markham *et al.* (2006).

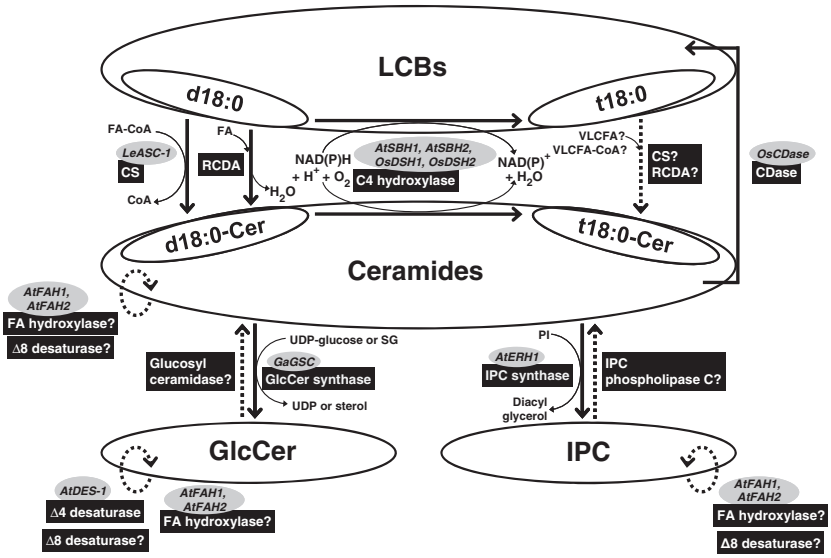

Fig. 3 Schematic representation of the sphingolipid biosynthesis pathway in plants. All metabolic steps indicated by a plain arrow have been demonstrated *in vitro*. Activities are indicated in black boxes. Genes that have been cloned are indicated in dark gray ovals. The substrates for desaturation and fatty acid (FA) hydroxylase remain to be determined. Although ceramidase (CDase) activity has been detected, the substrate specificity remains to be characterized. Note that reverse CDase activity (RCDA) and CDase are activities of the same enzyme. LCB, long-chain base; IPC, inositolphospho ceramide; VLCFA, very long chain fatty acid; SBH, sphingoid base hydroxylase.

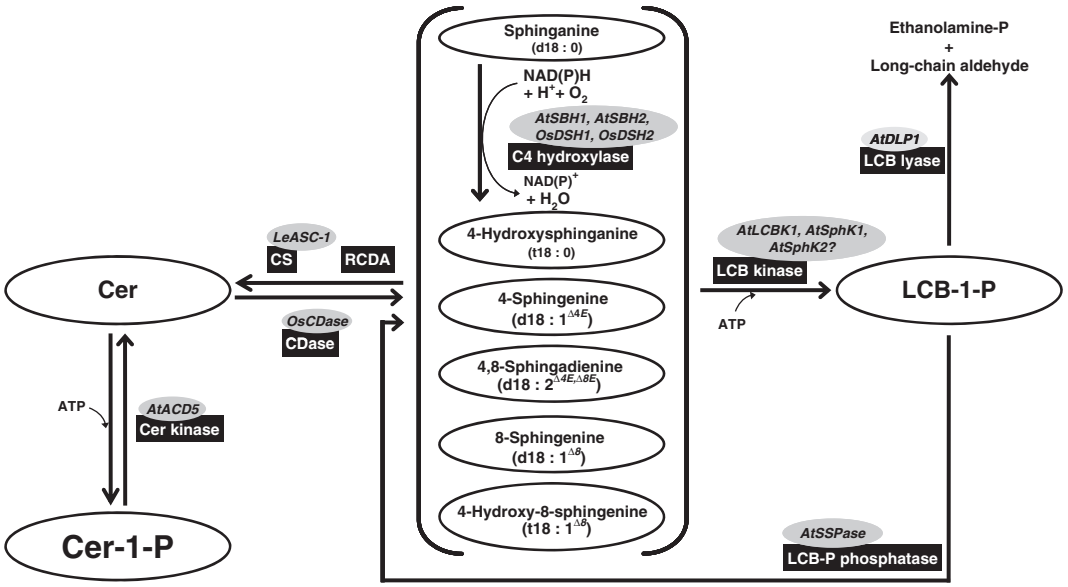

Fig. 4 Schematic representation of the sphingolipid metabolic pathway for phosphorylation of ceramides and long-chain bases (LCBs) in plants. Activities are indicated in black boxes. Genes that have been cloned are indicated in dark gray ovals. The substrate specificity has been characterized for LCB lyase and LCB kinase. Biochemical properties of LCB-P phosphatase remain to be determined.

The observation that ceramide may be involved in regulating programmed cell death in plants has led to efforts to understand how ceramides may be metabolized through the action of ceramidases. Much of our understanding of the function of ceramidases (E.C. 3.5.1.23) has been derived from work involving animal cells and yeast (Mao *et al.*, 2000a,b; Mao & Obeid, 2008). In animals, ceramidases are regarded as major regulators of ceramide-induced apoptosis (Choi *et al.*, 2003; Hannun & Obeid, 2008; Mao & Obeid, 2008) as they are key enzymes intimately involved in regulating the concentrations of ceramides and 4-sphingenine (sphingosine; d18:$1^{\Delta 4E}$), and hence sphingenine-1-

phosphate (S1P; d18:1$^{\Delta 4E}$-1-P). These three sphingolipid metabolites have been shown to be key bioactive mediators of cellular processes governing growth, differentiation, apoptosis and survival (Spiegel & Milstien, 2003; Hannun & Obeid, 2008; Mao & Obeid, 2008). Thus ceramidases are key enzymes regulating the availability of sphingosine and S1P, and thereby affecting the balance of ceramide/sphingenine/S1P in animal cells (Spiegel & Milstien, 2003; Hannun & Obeid, 2008; Mao & Obeid, 2008).

Ceramidases degrade ceramides by hydrolyzing the *N*-acyl linkage between the LCB and FA moieties. Diverse ceramidases have been characterized, differing in their subcellular localization, substrate specificities and pH optima. In general, ceramidases are classified according to their pH optima as acidic ceramidases, neutral ceramidases and alkaline ceramidases (Table 7). Readers are referred to Mao & Obeid (2008) for an excellent review of human ceramidases. The neutral ceramidase family is by far the best characterized of the three classes of ceramidases. They have been shown to be involved in key developmental processes in mammals. In human mesengial cells and rat hepatocytes, neutral ceramidase activity was shown to be modulated in response to various stimuli such as cytokines and growth factors, leading to control of cellular proliferation and differentiation (Coroneos *et al.*, 1995; Nikolova-Karakashian *et al.*, 1997). Knockdown of neutral ceramidases in zebrafish during embryogenesis has been shown to result in abnormal development, probably as a consequence of dysfunction in the circulatory system (Yoshimura *et al.*, 2004). Neutral ceramidases are considered as rate-limiting enzymes for the production of 4-sphingenine (d18:1$^{\Delta 4E}$). This free LCB is produced in animal cells only through the salvage pathway and not by *de novo* synthesis. Metabolism of ceramide by neutral ceramidases therefore makes an important contribution to the regulation of S1P (d18:1$^{\Delta 4E}$-1-P) signalling through the generation of 4-sphingenine (d18:1$^{\Delta 4E}$) (Mao & Obeid, 2008). It has therefore been suggested that neutral ceramidases are key modulators of cellular processes and signalling as they regulate the availability of the ceramide and LCB pools through their enzymatic activity (El Bawab *et al.*, 2002; Tani *et al.*, 2005; Mao & Obeid, 2008).

In contrast to the situation in animals and yeast, much less is known about ceramidases in plants. Lynch (2000) used a biochemical approach to show that a ceramidase activity can be isolated from plant membrane fractions. Optimal activity has been observed from pH 5.2 to 5.6 and Ca^{2+} is required as a cofactor. Sequence mining (BLAST) of the rice (*Oryza sativa*) genome using the human neutral ceramidase protein sequence showed the presence of one neutral ceramidase encoded by Os01g43520 (Pata *et al.*, 2008). The biochemical characterization of rice ceramidase established a broad pH optimum in the acidic to neutral range (Pata *et al.*, 2008; Table 7). This suggests that rice neutral ceramidase may have a variety of functions, as only one putative alkaline ceramidase with sequence similarities to the yeast phytoceramidases was found in the rice genome. Sequence analysis indicated that plants do not possess proteins with any sequence similarities to acid ceramidases.

Analysis of substrate utilization by rice ceramidase revealed a substrate preference for ceramide (d18:4$^{\Delta 4}$-ceramide) and not phytoceramide (t18:0-ceramide) (Pata *et al.*, 2008). This is interesting as d18:4$^{\Delta 4}$-ceramide is not the major ceramide species in fungi and plants (Ohnishi *et al.*, 1985; Sperling & Heinz, 2003; Dunn *et al.*, 2004; Lynch & Dunn, 2004). The substrate specificity of rice ceramidase for d18:4$^{\Delta 4}$-ceramide is consistent with it being a member of the neutral ceramidase family, as only the alkaline ceramidases have been shown to hydrolyze t18:0-ceramide (Mao & Obeid, 2008). Sphingolipidomic analyses of ceramide species following expression of rice ceramidase in the yeast double knockout mutant *Δypc1Δydc1*, which lacks the yeast ceramidases, also showed that both d18:0-ceramide (dihydroceramide) and t18:0-ceramide are unlikely to be substrates for rice ceramidase (Pata *et al.*, 2008). Reverse ceramidase activity (RCDA) has been reported in several members of the neutral ceramidase family (Table 7; Okino *et al.*, 1998; Kita *et al.*, 2000; El Bawab *et al.*, 2000; Wu *et al.*, 2007), and analyses of yeast sphingolipids following induction of rice ceramidase expression in the double knockout mutant *Δypc1Δydc1* showed elevated concentrations of t18:0-ceramide with FA chain lengths of C26 and C28 (Pata *et al.*, 2008). This observation suggests that rice ceramidase may exhibit RCDA, leading to elevated concentrations of these 2 t18:0-ceramide species (Fig. 4). The main route for ceramide formation is via ceramide synthase. For example, in plant preparations, RCDA is < 5% of ceramide synthase activity (Sperling & Heinz, 2003; Lynch & Dunn, 2004). For this reason, RCDA was proposed to occur to prevent cytotoxicity (Sperling & Heinz, 2003) or when ceramide synthase is inhibited (Mao *et al.*, 2000a,b).

In yeast, the *sur2* (sphinganine hydroxylase) gene encodes the enzyme responsible for the C4 hydroxylation of C18 and C20 LCBs. The C4 hydroxylase uses both d18:0-ceramide and free sphinganine (d18:0) as substrates (Dickson & Lester, 2002; Obeid *et al.*, 2002; Dickson *et al.*, 2006). Recently Chen *et al.* (2008) showed that a double T-DNA knockout of the *sur2* homologs (Sphingoid base hydroxylase; *SBH1* and *SBH2*) resulted in Arabidopsis plants lacking trihydroxy LCBs (4-hydroxysphinganine, t18:0 and 4-hydroxy-8-sphingenine, t18:1$^{\Delta 8}$). They also observed significant increases in total sphingolipid content, notably the contents of the complex sphingolipids and free LCBs (Chen *et al.*, 2008). In this mutant plant, complex sphingolipids and ceramides containing the C16 FA moiety appear to be elevated compared with complex sphingolipids and cera-

Table 7 Classification and characteristics of known neutral ceramidases

Source	Isoform	Molecular mass (kDa)	pH optimum	Substrate utilization	RCDA/pH RCDA optimum	Glycosylation	Subcellular localization	Predicted TM/ topogenesis	References
Mycobacterium tuberculosis		nd	~9	d18:1$^{\Delta4}$-Cer	?	High (N-glycosylation)	Cytosol	0/soluble	b
Pseudomonas aeruginosa		70	~7–8.5	d18:1$^{\Delta4}$-Cer = d18:0-Cer >> t18:0-Cer	+/~7		Extracellular, periplasm	1/secretory	a-e
Drosophila melanogaster		79	~6.5–7.5	d18:1$^{\Delta4}$-Cer = d18:0-Cer	+		Extracellular, periplasm		f, g
Dictyostelium discoideum		93	~3	d18:1$^{\Delta4}$-Cer = d18:0-Cer	+/~5		Extracellular, periplasm		h
Danio rerio			~7.5	d18:1$^{\Delta4}$-Cer	?	High (N-glycosylation)	ER, Golgi, PM, extracellular	1/type II integral; secretory	i
Mus musculus		94	~7–8.5	d18:1$^{\Delta4}$-Cer > d18:0-Cer	+	+ O-glycosylation on mucin-box[2]	PM, extracellular + endosome-like organelles (liver isoform)		j-m
Rattus norvigecus	Kidney	112	~5.5–7.5	d18:1$^{\Delta4}$-Cer >> d18:0-Cer	?		PM		n
	Liver	nd	?	?	?		Endosome-like organelles		n
	Intestine	116	~5.5–6.5	d18:1$^{\Delta4}$-Cer	?		?		o
	Brain	90–95	~7–10	αOH-d18:1$^{\Delta4}$-Cer >> d18:1$^{\Delta4}$-Cer >> d18:0-Cer	+/~6–7		?		p-r
Homo sapiens	Long isoform	142[1]	~7–8.5	d18:1$^{\Delta4}$-Cer	+		ER, Golgi, PM, extracellular		s, t
	Short isoform	140[1]	~7–9.5	d18:1$^{\Delta4}$-Cer	?		ER, Golgi, extracellular; mitochondrial		k, s
	Intestine	116	~6–8	d18:1$^{\Delta4}$-Cer	+/~5–7.5		?		u
Oryza sativa		nd	~5–7	d18:1$^{\Delta4}$-Cer			ER, Golgi		v

[1]Molecular mass (kDa) when expressed in HEK293 cells.
[2]Not for liver neutral ceramidase, localized in endosome-like organelles.
SphCER, sphingosine-ceramide; dihydroCER, dihydrosphingosine-ceramide; TM, transmembrane domain; ER, endoplasmic reticulum.
All enzymes have been cloned except for the rat and human intestine counterparts.
References: a, b, Okino *et al.* (1998, 1999)); c, Kita *et al.* (2000); d, Nieuwenhuizen *et al.* (2003); e, Wu *et al.* (2007); f, i, Yoshimura *et al.* (2002); g, Acharya *et al.* (2008); h, Monjusho *et al.* (2003); j, k, l, Tani *et al.* (2000a,b, 2005)); m, Romiti *et al.* (2000); n, Mitsutake *et al.* (2001); o, Olsson *et al.* (2004); p, q, r, El Bawab *et al.* (1999, 2000, 2002)); s, Hwang *et al.* (2005); t, Galadari *et al.* (2006); u, Ohlsson *et al.* (2007); v, Pata *et al.* (2008).
nd, not determined.

mides containing VLCFAs (≥ C20) (Chen *et al.*, 2008). The authors proposed the existence of an alternative ceramide synthesis pathway in plants using 4-hydroxysphinganine (t18:0) as the LCB moiety and VLCFA as the acyl donor (Chen *et al.*, 2008). Thus it is possible that the RCDA encoded by the neutral ceramidase homologs in plants, for example, rice ceramidase (Pata *et al.*, 2008), may be responsible for the 'alternative' ceramide synthesis pathway in plants.

III. Physiological functions of plant sphingolipid classes

1. Physiological functions of glycosyl inositol phosphoceramides (GIPCs)

In the late 1950s, Carter and colleagues focused their attention on inositol sphingolipids, which at that time were thought to be specific to the plant kingdom, and were named 'phytoglycolipids' (Carter *et al.*, 1958). The name 'phytoglycolipid' is now defunct and these compounds are now referred to as GIPCs (Spassieva & Hille, 2003; Worrall *et al.*, 2003; Lynch & Dunn, 2004). GIPCs are predominant forms of complex sphingolipids common to plants and fungi, but not present in animal cells (Obeid *et al.*, 2002; Warnecke & Heinz, 2003; Worrall *et al.*, 2003; Lynch & Dunn, 2004). Although GIPCs belong to one of the earliest classes of plant sphingolipids to be identified (Carter *et al.*, 1958; Carter & Koob, 1969; Kaul & Lester, 1975, 1978; Hsieh *et al.*, 1978, 1981), very few GIPCs have been fully characterized to date (Tables 1,2) because of their high polarity and relatively poor recovery using traditional extraction techniques (Spassieva & Hille, 2003; Sperling *et al.*, 2005; Markham *et al.*, 2006).

The cellular localization of a molecule is likely to provide insights into its function. Few data are available for the cellular localization of GIPCs, although it is widely assumed that they are localized to the plasma membrane (Worrall *et al.*, 2003; Lynch & Dunn, 2004; Sperling *et al.*, 2005). The relatively similar composition and proportion of LCBs between the plasma membrane and the GIPC fraction isolated from Arabidopsis leaves appear to lend credence to this assumption, although direct evidence for the cellular localization and transbilayer distribution of GIPCs in plant membranes is still lacking (Sperling & Heinz, 2003; Lynch & Dunn, 2004; Sperling *et al.*, 2005; Markham *et al.*, 2006; Markham & Jaworski, 2007).

The Arabidopsis IPC synthase ERH1 has been localized to the Golgi (Wang *et al.*, 2008), and sphingolipids have been shown to be involved in Golgi and ER integrity (Chen *et al.*, 2008). GIPCs have also been shown to be involved in early stages of symbiosis (Perotto *et al.*, 1995) and as GPI anchors of proteins preferentially partitioned into 'lipid rafts' (Bhat & Panstruga, 2005; Borner *et al.*, 2005). As such, GIPCs may be important determinants in cell signalling, cell-cell communication and the sorting of proteins, akin to the role of complex sphingolipids in animal development (Spassieva & Hille, 2003; Worrall *et al.*, 2003).

2. Physiological functions of glycosylceramides

Another class of plant sphingolipids are the glycosylceramides, which are structurally simpler than the GIPCs (Fig. 2). They are often referred to as 'cerebrosides' or 'glucocerebrosides' in the literature as they are structurally similar to galactosylceramide from the brain (Spassieva & Hille, 2003; Warnecke & Heinz, 2003). The glycosylceramides are also the most extensively characterized of the sphingolipid classes in terms of their structure, because they are abundant in the plant plasma and vacuolar membranes, and are relatively easy to extract and purify (Spassieva & Hille, 2003; Warnecke & Heinz, 2003; Lynch & Dunn, 2004; Takakuwa *et al.*, 2005). While GIPCs are unique to organisms possessing a cell wall (plants and fungi), glycosylceramides are common to most eukaryotic organisms and a few bacteria (Warnecke & Heinz, 2003).

In glycosylceramides, the headgroup hexoses are either β-glucose or β-mannose. In addition to the hexose moiety attached at C1, the FA moiety of plant glycosylceramides is usually α-hydroxylated (Lynch *et al.*, 1992; Norberg *et al.*, 1996; Whitaker, 1996; Sullards *et al.*, 2000; Bohn *et al.*, 2001). In many species, α-OH C16 is the major FA species. However, in the Poaceae family, the FA moieties of glycosylceramides are VLCFAs (FA with ≥ C20). Another characteristic of this family is the high concentrations of the ω9-desaturated FA moiety, especially α-OH C24:1 (Imai *et al.*, 2000). Tables 3 and 4 show the diversity and relative abundances of the VLCFAs and LCB moieties of glycosylceramides in plants (Tables 3,4).

Numerous functions have been ascribed to glycosylceramides in plants, and these include membrane stability, membrane permeability and pathogenesis. Glycosylceramides are present on the apoplastic monolayer of the lipid bilayer of plant plasma membranes. In this respect, their localization is similar to that of GIPCs, and they are exposed to the extracellular milieu (Spassieva & Hille, 2003; Warnecke & Heinz, 2003; Lynch & Dunn, 2004). Glycosylceramides have been implicated in chilling/freezing tolerance. For example, glycosylceramides containing α-OH monounsaturated VLCFAs appear to be detected mainly in chilling-resistant plants (Cahoon & Lynch, 1991; Imai *et al.*, 1995), and their concentrations have been shown to increase during cold acclimation in rye leaves (Lynch & Steponkus, 1987). By contrast, in chilling-sensitive plants, glycosylceramides containing saturated α-OH FA predominate (Imai *et al.*, 1995).

In addition to the FA moiety, the predominance of particular LCBs in the ceramide cores of glycosylceramides

may also be important during cold acclimation. Imai *et al.* (1997) demonstrated the predominance of the *cis* isomer of 4-hydroxy-8-sphingenine (t18:1$^{\Delta 8Z}$) relative to the *trans* isomer of 4-hydroxy-8-sphingenine (t18:1$^{\Delta 8E}$) in chilling-resistant plants (Imai *et al.*, 1997). It has been proposed that the involvement of glycosylceramides in cold acclimation is a result of their role in the cryostability of the membrane. The primary cause of freezing injury is dehydration-induced destabilization of the plasma membrane (Lynch & Steponkus, 1987; Uemura & Steponkus, 1994; Uemura *et al.*, 1995; Spassieva & Hille, 2003; Warnecke & Heinz, 2003) and glycosylceramides can affect cryostability in two main ways. Glycosylceramides can (1) influence monolayer fluidity via intramolecular and intermolecular lateral hydrogen bond formation, leading to a lower gel-to-liquid crystalline transition temperature, and (2) affect the intrinsic curvature of the plasma membrane, leading to reductions in the space between the plasma membrane and the endomembranes. This has the effect of increasing lipid de-mixing, which can result in a higher risk of drought-induced recombination and formation of fatal H_{II} phase. The lipid de-mixing can also affect the functionality of membrane proteins (Lynch & Steponkus, 1987; Cahoon & Lynch, 1991; Lynch *et al.*, 1992; Uemura & Steponkus, 1994; Uemura *et al.*, 1995; Spassieva & Hille, 2003; Warnecke & Heinz, 2003).

Glycosylceramides have also been implicated in drought tolerance, in addition to their role in maintaining membrane stability during cold acclimation (Warnecke & Heinz, 2003). For example, a lipidomic study of the vacuolar membrane of the leaves of a C3 and a Crassulacean acid metabolism (CAM)-induced *Mesembryanthenum crystallinum* plant (common ice plant) showed that the vacuolar membrane has a higher concentration of glycosylceramides in the drought-adapted (CAM) plant, suggesting a function in drought tolerance (Warnecke & Heinz, 2003). Similarly, when the glycosylceramide content of the plasma membrane of the resurrection plant *Ramonda serbica* (highly adapted to survive in water-deficit conditions) was examined, the results showed a higher glycosylceramide content (10.9 mol%) under dessicated conditions, and a lower glycosylceramide content (6.6 mol%) under hydrated conditions (Quartacci *et al.*, 2001).

In animal cells, glycosylceramides with a ceramide core consisting of a trihydroxy LCB (t18:0) and α-OH VLCFA have been shown to increase plasma membrane stability and reduce ion permeability. A similar function has also been proposed for plant glycosylceramides (Cahoon & Lynch, 1991; Lynch *et al.*, 1992; Lynch & Dunn, 2004). Aluminium (Al) is highly phytotoxic when present in excessive amounts in acidic soil and is a major limitation for plant growth. The phytotoxicity of Al is a result of its ability to induce programmed cell death in roots cells. Zhang *et al.* (1997) examined the effects of Al on two cultivars of wheat (*Triticum aestivum*) (the Al-sensitive Katepwa cultivar and the Al-resistant PT741 cultivar) and showed that, when plants were exposed to 20 μM $AlCl_3$ for 3 d, a slight increase in glycosylceramide content was observed in PT741 whereas the glycosylceramide content was reduced in Katepwa.

3. Physiological functions of ceramide

The third class of plant sphingolipids, the ceramides, are less well documented than the GIPCs and glycosylceramides. This may be attributed, in part, to their lower abundance in plant membranes compared with GIPCs and glycosylceramides. Vesper *et al.* (1999) estimated that the ceramide content of plant tissues is *c.* 10–20% of the glycosylceramide content, while Wang *et al.* (2006) observed a ceramide content in the range of 4–10 mol% of the glycosylceramide content. In rice and Arabidopsis, the ceramide content was estimated to be 6 mol% in the leafy stems of rice and between 2 and 7 mol% in Arabidopsis leaves (Ohnishi *et al.*, 1985; Markham *et al.*, 2006; Markham & Jaworski, 2007). Ceramides are relatively simple compared with GIPCs and glycosylceramides. A ceramide is formed by the *N*-acylation of an LCB and an FA (Fig. 2). The main FA moieties of plant ceramides are usually very long chain α-OH FAs, especially in Poaceae, although high proportions of some nonhydroxy FAs have also been found in rice and potato (*Solanum tuberosum*) (Table 5) (Fujino *et al.*, 1985; Ohnishi *et al.*, 1985; Bartke *et al.*, 2006). In addition to differences in the VLCFA component, plant ceramides also exhibit differences in the composition of the LCB. In general, the LCBs that predominate in plant ceramides are the trihydroxy-LCBs (Table 6) (Markham *et al.*, 2006; Markham & Jaworski, 2007).

Ceramide is a well-established inducer of apoptosis/programmed cell death (PCD) in animal cells (Hannun & Obeid, 2008). In plants, the first evidence for the involvement of ceramide in PCD was reported by Liang *et al.* (2003). For a discussion of plant PCD, readers are referred to excellent reviews by Lam (2004), Reape & McCabe (2008), and Reape *et al.* (2008). Liang *et al.* (2003) isolated and characterized the *accelerated cell death 5* (*acd5*) mutant of Arabidopsis and showed that ACD5 is a ceramide kinase. Knockout mutants of *acd5* showed more severe disease symptoms (hypersensitive lesions) during pathogen attack. The authors also showed that ceramide can induce PCD, while ceramide-induced PCD can be attenuated by ceramide-1-phosphate. *In vitro* activity assays using recombinant ACD5 showed that ceramide containing 4-sphingenine (d18:1$^{\Delta 4E}$) as the LCB moiety is a better substrate than ceramide containing sphinganine (d18:0) (Liang *et al.*, 2003). ACD5 does not appear to use diacylglycerol and 4-sphingenine (d18:1$^{\Delta 4E}$) as substrates, indicating that it is a *bona fide* ceramide kinase (Liang *et al.*, 2003). Recombinant ACD5 exhibited an alkaline pH optimum of 8.2 and an optimum temperature of 30°C (Liang *et al.*,

2003). The authors also showed that Ca^{2+} is a cofactor important for kinase activity. A role for ACD5 in pathogenesis was further supported by the observation that expression of *ACD5* was up-regulated when Arabidopsis plants were inoculated with a virulent strain of *Pseudomonas syringae* compared with an avirulent strain (Liang *et al.*, 2003).

The ability of ceramide to induce PCD was confirmed in another study by Townley *et al.* (2005), who showed that 50 μM C2-ceramide can induce PCD in Arabidopsis suspension cell cultures and that C2-ceramide-induced elevations in cytosolic-free Ca^{2+} ($[Ca^{2+}]_{cyt}$) act downstream of C2-ceramide. Interestingly, the authors showed that natural α-OH ceramides or synthetic ceramides with longer FA chain length (C6) were less able to induce PCD compared with C2-ceramide. Additionally, it was demonstrated that loss of potassium ions (K^+) is a key feature of ceramide-induced PCD in tobacco (*Nicotiana tabacum*) (Peters & Chin, 2007). More recent work has demonstrated that naturally occurring ceramides in Arabidopsis contain VLCFAs, and the finding that synthetic C2-ceramide can trigger more PCD than synthetic C6-ceramide suggests that hydroxyceramides with VLCFAs may not be active in regulating PCD. This is important, as PCD is a process that must be tightly regulated (Lam, 2004; Reape & McCabe, 2008; Reape *et al.*, 2008). It is envisioned that systematic analysis of naturally occurring ceramides will identify the ceramide subspecies that is important in regulating plant PCD.

4. Physiological functions of free LCBs

The fourth class of plant sphingolipids are the free LCBs. The structures of some free LCBs are shown in Fig. 1. The LCBs can also be phosphorylated (LCB-Ps) as part of their metabolism. The free LCBs and LCB-Ps are potentially interesting mediators of cellular responses (Ng *et al.*, 2001; Coursol *et al.*, 2003, 2005; Xiong *et al.*, 2008). One of the earliest indications that free LCBs may play a role in mediating cellular processes came from a study in oat (*Avena sativa*) mesophyll cells where free LCBs such as 4-sphingenine (d18:$1^{\Delta 4E}$), sphinganine (d18:0) and 4-hydroxysphinganine (t18:0) were shown to modulate redox activity (Dharmawardhane *et al.*, 1989). Interestingly, the authors reported that the effects of the free LCBs on redox activity were inhibitory and stimulatory in the dark and light, respectively. The significance of these results in a physiological context remains to be determined. Free LCBs such as 4-sphingenine (d18:$1^{\Delta 4E}$) may also regulate vacuolar pyrophosphatase (V-PPase) activity in *Chenopodium rubrum* suspension cell cultures (Bille *et al.*, 1992). V-PPases are proton pumps present in the vacuolar membrane which utilize inorganic pyrophosphate to regulate vacuolar and cellular acidity (Maeshima, 2001). Again, the physiological significance of the effects of sphingenine (d18:$1^{\Delta 4E}$) on V-PPase remains to be determined.

In mammalian cells, 4-sphingenine (d18:$1^{\Delta 4E}$), along with ceramide, has been shown to promote cell death. Brodersen *et al.* (2002) reported that the Arabidopsis *accelerated cell death 11* (*acd11*) mutant exhibits spontaneous cell death. Cloning of the *ACD11* gene revealed that it is similar to genes encoding mammalian glycolipid transfer proteins. In mammalian cells, these glycolipid transfer proteins have been shown to regulate the transport of complex sphingolipids across membranes. The authors showed that ACD11 can serve as a sphingosine transfer protein, but does not appear to transport complex sphingolipids (Brodersen *et al.*, 2002). By contrast, S1P (d18:$1^{\Delta 4E}$-1-P) has been shown to exhibit pro-survival activity (Spiegel & Milstien, 2003; Hannun & Obeid, 2008). The pro-survival activity of S1P (d18:$1^{\Delta 4E}$-1-P) can be attributed to its ability to suppress ceramide-induced cell death (Cuvillier *et al.*, 1996; Spiegel & Milstien, 2003). This has led to the suggestion that the dynamic balance of cellular concentrations of sphingolipid metabolites functions to regulate cell fate (acting as a 'cell death rheostat'), although the situation is likely to be highly complex because of the inter-convertibility of sphingolipid metabolites (Spiegel & Milstien, 2003; Futerman & Hannun, 2004; Hannun & Obeid, 2008).

Increases in free LCBs have been shown to be involved in PCD in plants. Tomato (*Solanum lycopersicum*) plants of the genotype *asc/asc* (*Alternaria* stem canker) are highly sensitive to AAL (*Alternaria alternata* f. sp. *lycopersici*) toxin produced by the nectrotrophic fungus *Alternaria alternata* f.sp. *lycopersici* (Wang *et al.*, 1996; Spassieva *et al.*, 2002). AAL toxin and other SAMs (sphinganine-analog mycotoxins) inhibit *de novo* ceramide synthesis. The associated accumulation of free LCBs, 4-hydroxysphinganine (t18:0) and more importantly sphinganine (d18:0) was proposed to lead to the induction of PCD (Wang *et al.*, 1996; Brandwagt *et al.*, 2000). Support for this hypothesis was provided by Spassieva *et al.* (2002), who showed that AAL-toxin-induced PCD can be attenuated by treating leaf discs with myriocin, an inhibitor of serine palmitoyltransferase (SPT), the enzyme responsible for the formation of 3-ketosphinganine, a precursor in the *de novo* pathway of sphingolipid synthesis. Myriocin was able to attenuate AAL-toxin-induced PCD by reducing the accumulation of free sphinganine (d18:0) (Spassieva *et al.*, 2002). Takahashi *et al.* (2009) reported that infection of *Nicotiana benthemiana* by the nonhost pathogen *Pseudomonas cichorii* caused the accumulation of the mRNA for the LCB2 subunit of SPT and that treatment with myriocin compromised nonhost resistance.

Shi *et al.* (2007) reported that an Arabidopsis fumonisin B1 (FB1)-resistant mutant (*fbr11-1*) was unable to accumulate free LCBs and to undergo PCD in the presence of FB1. The authors showed that direct feeding of sphinganine (d18:0), 4-hydroxysphinganine (t18:0), and 4-sphingenine (d18:$1^{\Delta 4E}$) to Arabidopsis suspension cell cultures resulted

in reactive oxygen species (ROS) production, leading to cell death. Interestingly, the authors also demonstrated that sphinganine-1P (d18:0-1-P) could attenuate sphinganine (d18:0)-induce ROS production and PCD (Shi *et al.*, 2007). Tsegaye *et al.* (2007) also investigated the effects of FB1 on Arabidopsis plants and showed that mutants of LCB phosphate lyase (responsible for the breakdown of LCB phosphates) are hypersensitive to FB1 compared with wild-type plants. The authors showed conclusively that At-DPL is a *bona fide* LCB phosphate lyase and that mutants of LCB phosphate lyase also accumulated more 4-hydroxy-8-sphingenine-1-P (t18:1-1-P) compared with wild type. Together, these studies support the involvement of LCB metabolism in regulating responses to pathogens.

In addition to their potential role as cell fate mediators, LCB-Ps have also been shown to be key signalling intermediates in the regulation of stomatal apertures (Ng *et al.*, 2001; Coursol *et al.*, 2003). S1P (d18:1$^{\Delta 4E}$-1-P) has long been established as an anti-apoptotic agent in mammalian cells, and has been reported to be involved in various cellular responses such as proliferation, cytoskeleton organization and differentiation (Cuvillier *et al.*, 1996; Pyne & Pyne, 2000; Spiegel & Milstien, 2003). The bioactivity of S1P (d18:1$^{\Delta 4E}$-1-P) has been attributed to its ability to mobilize Ca^{2+} in mammalian cells (Mattie *et al.*, 1994). Ng *et al.* (2001) provided the first evidence for the presence of S1P (d18:1$^{\Delta 4E}$-1-P) in *Commelina communis* and showed that S1P (d18:1$^{\Delta 4E}$-1-P) is able to induce reductions in stomatal apertures in a dose-dependent manner in *C. communis*. More recently, Michaelson *et al.* (2009) found trace levels of 4-sphingenine (d18:1$^{\Delta 4E}$) in *C. Commelina.* Ng *et al.* (2001) also showed that sphinganine-1P (d18:0-1-P) was not able to cause any change in stomatal aperture. These results indicate that bioactivity of S1P (d18:1$^{\Delta 4E}$-1-P) is conferred by the Δ4 double bond. Ng *et al.* (2001) also showed that the effects of S1P (d18:1$^{\Delta 4E}$-1-P) on stomatal aperture are Ca^{2+}-dependent, as the calcium chelator EGTA was able to inhibit the effects of S1P (d18:1$^{\Delta 4E}$-1-P). They also provided direct evidence that S1P (d18:1$^{\Delta 4E}$-1-P) can cause elevations in the cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]_{cyt}$), in the form of oscillations. Additionally, they were able to attenuate abscisic acid (ABA)-induced reductions in stomatal aperture with DL-*threo*-dihydrosphingosine, a competitive inhibitor of sphingosine kinase (SphK). These results suggest that ABA may regulate the activity of SphK, thereby modulating the endogenous concentration of S1P (d18:1$^{\Delta 4E}$-1-P) in stomatal guard cells (Ng *et al.*, 2001).

The results obtained by Ng *et al.* (2001) were corroborated by Coursol *et al.* (2003, 2005) using Arabidopsis. Coursol *et al.* (2003) showed that ABA can activate SphK rapidly (within 2 min) and that ABA activation of SphK can be inhibited by the SphK inhibitors DL-*threo*-dihydrosphingosine and *N,N*-dimethylsphingosine. These authors showed that S1P (d18:1$^{\Delta 4E}$-1-P) can inhibit the inward-rectifying K^+ channels (important for stomatal opening) and activate the slow anion channels (important for stomatal closure). The combined effects of S1P (d18:1$^{\Delta 4E}$-1-P) on the ion channels result in a reduction in the turgor of the pair of guard cells that flank each stomatal pore, leading to reductions in stomatal aperture (Coursol *et al.*, 2003). Interestingly, the authors also showed, using knockout mutants of the α-subunit of heterotrimeric G proteins (*gpa1*), that ABA activation of SphK activity and S1P (d18:1$^{\Delta 4E}$-1-P) regulation of ion channel activities are mediated in part through the heterotrimeric G proteins (Coursol *et al.*, 2003).

More recently, Michaelson *et al.* (2009) characterized a *bona fide* Δ4-desaturase from Arabidopsis and showed that it catalyzes the Δ4-desaturation of glycosylceramides. Interestingly, they showed that the Arabidopsis Δ4-desaturase gene is specifically expressed in floral tissues and that knockout mutants do not appear to exhibit any observable phenotypic differences compared with wild type (transpirational water loss from detached leaves, ABA regulation of stomatal apertures, and pollen germination), leading the authors to question the importance of 4-sphingenine (d18:1$^{\Delta 4E}$) and S1P (d18:1$^{\Delta 4E}$-1-P) in Arabidopsis (see also review by Lynch *et al.*, 2009).

Interestingly, Coursol *et al.* (2005) showed that 4-hydroxysphinganine-1P (t18:0-1-P) can also inhibit stomatal opening and promote stomatal closure and that the effects of 4-hydroxysphinganine-1P (t18:0-1-P) are also mediated through heterotrimeric G proteins. This raises interesting questions, as only trace levels of S1P (d18:1$^{\Delta 4E}$-1-P) have been detected in plant extracts. It is possible that the bioactivity exhibited by S1P (d18:1$^{\Delta 4E}$-1-P) and 4-hydroxysphinganine-1P (t18:0-1-P) relates to the molecular structure of the LCBs (Fig. 5). The bioactivities of sphingenine (d18:1$^{\Delta 4E}$) and S1P (d18:1$^{\Delta 4E}$-1-P) have been proposed to be attributable to the presence of the Δ4 double bond (position C4) (Radin, 2004). Analysis of the structure of S1P (d18:1$^{\Delta 4E}$-1-P) indicated the presence of an allylic alcohol group composed of the Δ4 double bond (position C4) and a hydroxyl group at C3 (Fig. 5). This allylic alcohol group is absent in sphinganine-1P (d18:0-1-P). Examination of the molecular structure of 4-hydroxysphinganine-1P (t18:0-1-P) showed the presence of a hydroxyl group at C4. It is possible that the hydroxyl group at C4 in 4-hydroxysphinganine-1P (t18:0-1-P) can confer bioactivity similar to that conferred by the presence of the allylic alcohol group in S1P (d18:1$^{\Delta 4E}$-1-P). The ability of a functional hydroxyl group at C4 to confer bioactivity was demonstrated by Hwang *et al.* (2001), who showed that substituting the Δ4-double bond of ceramide with a hydroxyl group at C4 resulted in a greater potency for induction of apoptosis in SK-N-BE(2)C and N1E-115 cells. This may provide a plausible explanation for the observed bioactivity of S1P

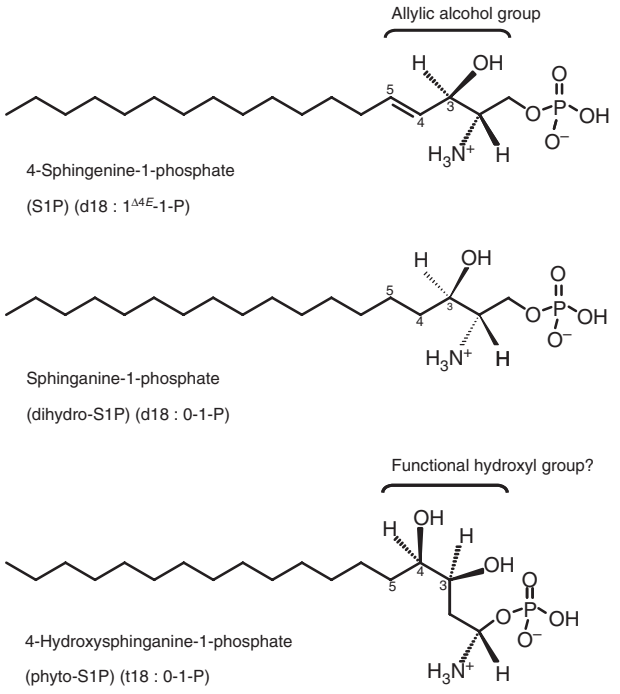

Fig. 5 Schematic representation of the structure of the long-chain base phosphates 4-sphingenine-1-phosphate (S1P; d18:1$^{\Delta 4E}$-1-P), sphingahine-1-phosphate (dihydro-S1P; d18:0-1-P), and 4-hydroxysphinganine-1-phosphate (phyto-S1P; t18:0-1-P). The allylic alcohol group of d18:1$^{\Delta 4E}$-1-P comprising the hydroxyl group at C3 and the Δ4 double bond (C4) is shown. The positions of the carbon atoms are shown as numbers (only positions 3–5 are indicated). In 4-hydroxysphinganine-1-phosphate, a hydroxyl group is present at C4 as opposed to a Δ4 double bond which is characteristic of d18:1$^{\Delta 4E}$-1-P.

(d18:1$^{\Delta 4E}$-1-P) and 4-hydroxysphinganine-1P (t18:0-1-P) in stomatal guard cells of Arabidopsis.

The results obtained using a pharmacological approach (Ng *et al.*, 2001; Coursol *et al.*, 2003, 2005) have recently been corroborated by Worrall *et al.* (2008), who reported the cloning of sphingosine kinase 1 (SphK1) from Arabidopsis. The authors showed conclusively that SphK1 is involved in mediating ABA regulation of stomatal aperture and seed germination. Nishikawa *et al.* (2008) showed, using transpirational water loss from detached leaves, that mutants of LCB phosphate lyase exhibit slower rates of water loss. The pool of LCB-Ps appears to modulate the overall process of stomatal regulation and/or dehydratation stress, because the LCB-P catabolism enzymes S1P lyase (AtDLP1) and S1P phosphatase (AtSSPase) may also be involved in this process (Nishikawa *et al.*, 2008; Worrall *et al.*, 2008). Together, these studies highlight the importance of LCBs and LCB-Ps as key mediators of cellular processes in plant cells. It is envisioned that more research will lead to greater understanding of the roles these sphingolipid metabolites may play in plant cells.

Long-chain bases with the Δ8 double bond are typical of plants and many fungi. Sperling *et al.* (1998) identified two orthologs of the sphingolipid Δ8-desaturase with an N-terminal cyt b_5 domain in Arabidopsis and rapeseed (*Brassica napus*). The cyt b5 domain functions as an intermediate electron donor in many acyl desaturases (Sperling *et al.*, 1998; Spassieva & Hille, 2003), and is often associated with the ER in plants (Zhao *et al.*, 2003). A sphingolipidomic study of yeast expressing the Arabidopsis or *Brassica* sphingolipid Δ8-desaturases demonstrated the production of 4-hydroxy-8-sphingenine (t18:1$^{\Delta 8}$) (Sperling *et al.*, 1998). Expression of the sphingolipid Δ8-desaturase homologs from sunflower (*Helianthus annuus*) and *Borago officinalis* in yeast also produced the same results (Sperling *et al.*, 2000, 2001). Further investigations using the hydroxylase mutant strain *sur2Δ* allowed the authors to conclude that the substrate of the sphingolipid Δ8-desaturases is 4-hydroxysphinganine (t18:0) (Sperling *et al.*, 2000). As sphinganine (d18:0) does not appear to be a substrate, the Δ8-desaturation reaction was therefore proposed to be downstream of the C4 hydroxylation (Sperling *et al.*, 2000).

Recently, the characterization of the sphingolipid Δ8-desaturase from *Stylosanthes hamata* showed that it has a substrate preference for the *cis*-isomer of 4-hydroxysphinganine (Ryan *et al.*, 2007). It is unclear how 4-hydroxysphinganine (t18:0) is presented to the sphingolipid Δ8-desaturase, although it has been suggested that glycosylceramides may be substrates (Ryan *et al.*, 2007). da Silva *et al.* (2006) and Ryan *et al.* (2007) found that expression in yeast of the sphingolipid Δ8-desaturases from *Brassica napus*, Arabidopsis, *Helianthus annuus* or *Stylosanthes hamata* conferred tolerance to cytotoxic concentrations of Al. When the *S. hamata* sphingolipid Δ8-desaturase was overexpressed in Arabidopsis, it conferred increased Al tolerance in 2-wk-old seedlings and enhanced root growth in the presence of Al concentrations ranging from 300 to 500 μM (Ryan *et al.*, 2007). This is contrast to the observation that maize (*Zea mays*) transgenic lines expressing the Arabidopsis sphingolipid Δ8-desaturase exhibited enhanced Al sensitivity compared with the parental lines (da Silva *et al.*, 2006). This may be attributable to the production of 8 times more *trans*-isomers of 4-hydroxy-8-sphingenine (t18:1$^{\Delta 8E}$) in the transgenic lines than in the parental lines, catalyzed by the Arabidopsis sphingolipid Δ8-desaturase, whereas the sphingolipid Δ8-desaturase from *S. hamata* preferentially forms *cis*-isomers of 4-hydroxy-8-sphingenine (t18:1$^{\Delta 8Z}$) (da Silva *et al.*, 2006; Ryan *et al.*, 2007). These observations led the authors to suggest that the stereospecificity of the sphingolipid Δ8-desaturase may be an essential feature of resistance or sensitivity to Al phytotoxicity.

IV. Conclusion

Plant sphingolipids are no longer as enigmatic as they were 10–20 yr ago. A better understanding of the roles that sphingolipids play in plant physiology will come from bet-

ter understanding at the levels of transcriptomics, proteomics and, perhaps most importantly, the sphingolipidome. It is envisioned that a clearer picture of the essential roles of plant sphingolipids will emerge as more plant sphingolipidomes are characterized. The coordinated effort to develop community-based standards and protocols along the lines of the LipidMaps consortium (http://www.lipidmaps.org/) will provide an important way forward for the plant sphingolipid community.

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