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1 **Sphingolipids: towards an integrated view of metabolism during the**
2 **plant stress response**

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28 **Summary**

29 Plants exist in an environment of changing abiotic and biotic stresses. They have developed a
30 complex set of strategies to respond to these stresses and over recent years it has become
31 clear that sphingolipids are a key player in these responses. Sphingolipids are not universally
32 present in all three domains of life. Many bacteria and archaea do not produce sphingolipids
33 but they are ubiquitous in eukaryotes and have been intensively studied in yeast and
34 mammals. During the last decade there has been a steadily increasing interest in plant
35 sphingolipids. Plant sphingolipids exhibit structural differences when compared to their
36 mammalian counterparts and it is now clear that they perform some unique functions.
37 Sphingolipids are recognized as critical components of the plant plasma membrane and
38 endomembrane system. Besides being important structural elements of plant membranes,
39 their particular structure contributes to the fluidity and biophysical order. Sphingolipids are
40 also involved in multiple cellular and regulatory processes including vesicle trafficking, plant
41 development and defense. This review will focus on our current knowledge as to the function
42 of sphingolipids during plant stress responses, not only as structural components of biological
43 membranes, but also as signaling mediators.

44 **Key words:** sphingolipid, biotic stress, abiotic stress, programmed cell death, pathogens,
45 plant defense

46

47 **Introduction**

48 The strategies plants employ to endure stressful conditions are varied and involve a multitude
49 of molecular, metabolic and physiological adaptations. There is now a significant body of work
50 to indicate that sphingolipids are an important part of the arsenal of tools the plant has at its
51 disposal to respond to stress. Sphingolipids are an incredibly diverse group of compounds
52 (Pata *et al.*, 2010) with a vast array of physical properties which facilitate their function in a
53 variety of cellular processes. Sphingolipids form a significant proportion of the lipids present in
54 higher plants. Studies suggest sphingolipids constitute up to 40% of lipids in the plasma
55 membrane of plant cells (Cacas *et al.*, 2016) and are enriched in the endosomes and
56 tonoplasts (Moreau *et al.*, 1998). More comprehensive extraction techniques have been
57 developed over recent years which when coupled with technological advances in mass
58 spectrometry and chromatography have allowed improved sphingolipid identification and the

59 discovery of novel structures from smaller quantities of material (Cacas *et al.*, 2016). This has
60 enabled research to determine the contribution sphingolipid metabolites make in different
61 cellular processes.

62 An overview of the sphingolipid biosynthetic pathway is presented in Fig. 1. The term
63 sphingolipid covers a class of lipids whose defining component is a long-chain or sphingoid
64 base (LCB; for ease of reference, Table S1 lists the abbreviations used in this review). The
65 LCB is a carbon amino-alcohol backbone most commonly of 18 carbons which is synthesized
66 by the condensation of serine and palmitoyl-CoA catalysed by serine palmitoyl transferase
67 (SPT) in the endoplasmic reticulum (ER) (Chen *et al.*, 2006). The product of this reaction, 3-
68 ketosphinganine, is then reduced by the action of the 3-ketosphinganine reductase to
69 sphinganine (d18:0) (Beeler *et al.*, 1998). The LCB is considered the simplest functional
70 sphingolipid and can have a range of modifications including phosphorylation, desaturation
71 and hydroxylation. It is sometimes referred to as the free LCB. The LCB may be linked to a
72 very long chain fatty acid *via* an amide bond to form a ceramide. The fatty acyl component is
73 usually 16–26 carbons. This reaction is catalyzed by ceramide synthase. In *Arabidopsis*
74 *thaliana* (hereafter *Arabidopsis*) three ceramide synthases have been identified, LOH1–3.
75 Ceramidases catalyse the reverse reaction and are a component in regulating the ceramide
76 pool and sphingolipid homeostasis (Pata *et al.*, 2008). Ceramides can be phosphorylated in
77 the ER by ceramide kinases (CerK) or ACD5 (accelerated cell death 5) or further modified to
78 form the complex sphingolipids glycosylceramides (GlcCers) in the ER and glycosyl-
79 inositolphosphorylceramides (GIPCs) by the addition of simple or multiple sugars on
80 ceramide at the C1 position in the Golgi. These reactions are catalyzed by glucosylceramide
81 synthase (GCS) and at least three functional IPC-synthases and several glycosyl or
82 glucuronyl transferases (Wang *et al.*, 2008; Mina *et al.*, 2010; Rennie *et al.*, 2014; Msanne *et*
83 *al.*, 2015). The complex sphingolipids can exhibit very high levels of sugar decoration. One
84 study of 23 plant species identified at least 21 different patterns showing variation in number,
85 type and order of glycan substitutions (Cacas *et al.*, 2013). The biosynthesis of complex
86 sphingolipids is tightly controlled and the GIPC pool is regulated by the hydrolysis of GIPC to
87 phytoceramide-1 phosphate by the action of a phospholipase D (PLD) (Tanaka *et al.*, 2013).
88 Functional characterizations of enzymes of the sphingolipid biosynthetic pathway have also
89 pointed to the controls on the pathway and the specific pool sizes and structures that are
90 generated. This flexibility enables sphingolipids to constitute both a structural membrane
91 component and a signaling molecule from the same basic lipid backbones. For more details
92 about sphingolipid biosynthesis, see the recent reviews by Luttgeharm *et al.*, 2016,
93 Michaelson *et al.*, 2016 and Mamode Cassim *et al.*, 2019.

94 In plants, the size of the different sphingolipid pools tends to vary in a species and tissue-
95 dependent manner. For example, the occurrence of the LCB d18:2 containing GlcCer in
96 Arabidopsis is mainly confined to floral and pollen tissue (Michaelson *et al.*, 2009) and
97 sphingolipid distribution changes during fruit development and ripening (Ines *et al.*, 2018).
98 However outside of the Brassicaceae family d18:2 production, occurs throughout the plant,
99 and in species such as tomato and soybean is the most abundant GlcCer (Markham *et al.*,
100 2006). Wheat was found to contain much higher levels of d18:1 in its LCBs when compared
101 with rice (Goto *et al.*, 2012). In addition, the different tissues in rice have been found to
102 contain a similar quantity of sphingolipids but distribution across the lipid classes altered. A
103 survey of 21 different plant species from different phylogenetic groups found d18:1 ^{Δ4} to be
104 present in non-seed land plants and monocots but absent from Arabidopsis and soybean
105 (Islam *et al.*, 2012).

106

107 The functional significance of variations in sphingolipid chemical diversity and abundance is
108 still in the early stages of investigation. The different classes and modifications offer a variety
109 of differing solubility, charge, shape, and size. It is this array of properties which confer the
110 potential of sphingolipids to function both as bio-active components of cells involved in
111 regulating cellular processes and as integral components involved in the structural integrity of
112 the membranes. Regulation of sphingolipid metabolism enables plants to facilitate cell growth
113 and to appropriately respond to stress, both biotic and abiotic, using different metabolites to
114 modulate its response.

115

116 Here, we summarize our current knowledge on the role of sphingolipids in plants in response
117 to environmental cues and stress.

118

119 **Signals in programmed cell death**

120 Recent work utilizing genetically altered plants and plants exposed to sphingolipid
121 biosynthesis inhibitors have revealed that sphingolipids are regulators of programmed cell
122 death (PCD) occurring either during plant development or immunity. Perception of a stress
123 often occurs at the plasma membrane level. Therefore its integrity is essential for cell
124 signaling and survival. Sphingolipids are major structural constituents of plant plasma
125 membrane microdomains and their relationship with other components of the plasma
126 membrane is crucial. Changes in sphingolipid biosynthesis thus impact the microdomain
127 composition and this could affect protein content and distribution due to altered interactions

128 between plasma membrane components. For example, Bax-inhibitor-1 (AtBI-1, an inhibitor of
129 Bax-induced cell death) interacts with both FAH1 and FAH2 (fatty acid 2-hydroxylase). Plants
130 overexpressing AtBI-1 thus displayed enrichment in 2-hydroxy fatty acid-containing GlcCer in
131 microdomains as well as a loss of two proteins usually specifically localized to microdomains
132 (Ishikawa *et al.*, 2015). These two proteins feature in plant defense, both being involved in
133 cell death triggered by salicylic acid (SA) or oxidative stress. This reduction in protein content
134 led to an enhanced tolerance to SA or oxidative stress in AtBI-1 overexpressing plants
135 (Ishikawa *et al.*, 2015). These data suggest that the integrity of microdomains is critical to cell
136 death and sphingolipids are central to these structures.

137

138 Sphingolipids are involved in the control of PCD either as structural components of
139 membranes but also as initiators in the cell death regulatory pathway. The existence of a
140 rheostat between ceramides/LCBs and their phosphorylated counterparts already described
141 in animal cells is thought to exist in plants and similarly to control cell fate. According to this
142 model, ceramides and LCBs are able to trigger cell death whereas ceramide phosphates and
143 LCB-Ps promote cell survival (Shi *et al.*, 2007; Alden *et al.*, 2011) (Fig. 2). The induction of
144 PCD by LCB was based on the activation of protein kinases, MPK6 (Saucedo-Garcia *et al.*,
145 2011) or 14-3-3-regulated CPK3 (Lachaud *et al.*, 2013). The spontaneous PCD observed in
146 the *acd5* mutant, defective in ceramide kinase and with enhanced levels of ceramides, was
147 due to a strong accumulation of mitochondrial reactive oxygen species (ROS) (Bi *et al.*,
148 2014). This suggests that ROS are component of sphingolipid-induced PCD. The mycotoxin
149 fumonisin B1 (FB1) has been widely used to study both sphingolipid biosynthesis and PCD.
150 Indeed, FB1 is a strong inhibitor of ceramide synthase and has been shown to induce PCD.
151 When applied to plants, FB1 also triggered the accumulation of LCBs and LCB-Ps (Shi *et al.*,
152 2007; Tsegaye *et al.*, 2007; Saucedo-Garcia *et al.*, 2011; Yanagawa *et al.*, 2017).
153 Overexpression of *AtLCBK1* (Arabidopsis sphingoid- LCB kinase) in plant induced resistance
154 to FB1 treatment and conversely, *AtLCBK1* knockdown plants, exhibited a sensitivity to such
155 a treatment (Yanagawa *et al.*, 2017). Moreover, the authors demonstrated that transgenic
156 alteration of proteins involved in the LCB/LCB-P homeostasis (*AtLCBK1*, *AtSPP1* and
157 *AtDPL1*) resulted in a positive correlation between the levels of free LCBs and the degree of
158 FB1-induced cell death (Yanagawa *et al.*, 2017).

159

160 Increase in SPT activity, by overexpression of *AtssSPTa*, (small subunit of SPT) resulted in
161 an accumulation of LCBs and reduced tolerance to FB1 whereas *AtssSPTa* suppression
162 lines displayed lower levels of LCBs but enhanced tolerance to FB1 (Kimberlin *et al.*, 2013).
163 It was recently demonstrated by two independent studies that orosomucoid-like proteins
164 *AtORM1* and *AtORM2* physically interact with the core SPT complex and function as

165 repressor of SPT activity (Kimberlin *et al.*, 2016; Li *et al.*, 2016). ORM proteins thus regulate
166 sphingolipid homeostasis by differently modulating functionally different ceramide synthase
167 activities (Kimberlin *et al.*, 2016). *AtORM1* and *AtORM2* overexpressing plants were more
168 tolerant to FB1 treatment when compared to wild-type (WT) plants. This tolerance is
169 accompanied by a lower accumulation of C16 ceramides, LCBs and their phosphorylated
170 counterparts. Conversely, *AtORM* RNAi lines were more sensitive to such treatment, and
171 displayed higher content of C16 ceramides, LCBs and LCB-Ps (Kimberlin *et al.*, 2016).
172 Similarly, the ceramide synthase *LOH2* overexpressing lines resulted in the accumulation of
173 ceramides containing C16 fatty acids and dihydroxy LCBs and had reduced accumulation of
174 free LCBs and LCB-Ps in response to FB1. This overexpression also resulted in constitutive
175 induction of PCD and increased resistance to FB1 (Luttgeharm *et al.*, 2015). These findings
176 suggested that FB1-induced PCD is primarily due to the accumulation of free LCBs rather
177 than the accumulation of ceramides containing C16 fatty acids/dihydroxy LCBs. Curiously,
178 growth and increased cell division were promoted in *LOH1* and *LOH3* overexpressing plants,
179 which displayed enhanced production of ceramides with very long chain fatty acids (VLCFAs)
180 and trihydroxy LCBs (Luttgeharm *et al.*, 2015). These unexpected outcomes for growth and
181 development could be due to a ceramide synthesis with a certain chain length fatty acid and
182 quantity and in response to the correct stimuli. It is also known that VLCFA-ceramides are
183 important for Golgi trafficking and cell plate or phragmoplast formation during cell division in
184 Arabidopsis (Molino *et al.*, 2014). It is thus possible that increased cell expansion could be
185 due to a sphingolipid targeting to plant membranes that contributes directly to cell expansion.
186 In addition, the fatty acid hydroxylase double mutant *fah1/fah2* fails to form spontaneous
187 lesions under standard culture conditions despite an accumulation of free trihydroxy LCBs,
188 C16- and VLCFA-ceramides and SA (König *et al.*, 2012). Moreover, the *gonst1* (golgi
189 localized nucleotide sugar transporter1, involved in glycosylation of GIPCs) mutant displayed
190 spontaneous hypersensitive reaction (HR)-like lesions but did not accumulate ceramides or
191 LCBs (Mortimer *et al.*, 2013). One potential explanation for the differences that have been
192 observed is that several different mechanisms could be responsible for inducing cell death.

193

194 **Sphingolipids as structural components in response to abiotic stress**

195

196 Several studies have recently reported a role of sphingolipids in response to a temperature
197 stress. Acclimation capacity was correlated with changes in the content of TAGs
198 (triacylglycerols), MGDG (monogalactosyldiacylglycerol), DGDG (digalactosyldiacylglycerol)
199 and a GlcCer (Degenkolbe *et al.*, 2012). Analysis of oat, rye and Arabidopsis lipid profiles

200 during cold acclimation demonstrated that GlcCer contents decreased in the plasma
201 membrane whereas they were unchanged in microdomains (Minami *et al.*, 2009; Takahashi
202 *et al.*, 2016). These changes could contribute to a greater hydration of the plasma membrane
203 that could, in turn, increase membrane stability during cold stress. In a study focusing on
204 grapevine leaves, it was found that high levels of t18:1 (8Z) in complex sphingolipids were
205 correlated with freezing tolerance (Kawaguchi *et al.*, 2000). The sphingolipid $\Delta 8$ long-chain
206 base desaturases (SLD), which desaturate the LCB at the $\Delta 8$ position in both cis and trans
207 orientation, appear to play a role in cold tolerance in Arabidopsis (Chen *et al.*, 2012) and
208 tomato (Zhou *et al.*, 2016). In Arabidopsis, the *sld1sld2* double mutant is sensitive to cold
209 stress (Chen *et al.*, 2012). Similarly, *SISLD* knock-down tomato plants displayed greater
210 membrane damage and physiological indicators of chilling damage after stress than WT
211 plants. Chloroplasts are the main organelle impacted by cold and many studies have reported
212 that chloroplast morphology is affected by changes in lipid unsaturation. Chloroplasts in
213 *SISLD* knockdown were more severely damaged than in WT and the surviving organelles
214 were not surrounded by an extra-membrane (Zhou *et al.*, 2016). GlcCers, believed to stabilize
215 membranes, were detected in the envelope membrane of chloroplasts (Spassieva and Hille,
216 2003), suggesting that sphingolipids are structurally important for chloroplast membrane for
217 cold tolerance. This illustrated that disrupting *SISLD* transcript accumulation reduced chilling
218 tolerance of tomato. Lipid desaturation is a way for plants to mitigate the effects of chilling or
219 freezing temperatures. The *SISLD* knockdown plant sensitivity to chilling could thus be related
220 to the membrane properties such as fluidity that is diminished due to depletion of
221 sphingolipids with unsaturated LCBs. Another explanation for the decrease in cold tolerance
222 could be a change in the formation and content of microdomains in the membrane. It is
223 conceivable that activity of some microdomain-localized proteins important for cold tolerance
224 could be modified in perturbed microdomains (Chen *et al.*, 2012). There has been no
225 characterized function for sphingolipids in tolerance of high temperature in contrast to the
226 high concentration of trienoic fatty acids in the thylakoid membranes which have been shown
227 to be involved in both chilling and high temperature tolerance (Murakami *et al.*, 2000;
228 Routaboul *et al.*, 2012; Tovuu *et al.*, 2016).

229

230

231 **Sphingolipids as structural components in response to biotic stress**

232

233 The rice *Osfah1/2* plants displayed similar SA levels to WT and a decreased tolerance to the
234 hemibiotrophic fungus *Magnaporthe oryzae*. Nagano and colleagues demonstrated that
235 products of these enzymes, 2-hydroxy-sphingolipids, were critical in the formation of

236 microdomains and disruption of OsFah1/2 activity disturbed organization of defense proteins
237 localized in these microdomains, such as the NADPH oxidase RbohB, required for ROS
238 production involved in rice immunity (Nagano *et al.*, 2016).

239

240 Recent work has identified three genes involved in GIPC glycosylation: GONST1, IPUT1
241 (inositol phosphorylceramide glucuronosyltransferase1) and GMT1 (GIPC mannosyl-
242 transferase1) (Mortimer *et al.*, 2013; Fang *et al.*, 2016; Tartaglio *et al.*, 2017). These three
243 mutants displayed high SA and ROS levels coupled to a constitutive HR and defense-gene
244 induction, suggesting a constitutive biotic stress response. Interestingly, *gmt1* also had a
245 decrease in cellulose accompanied by an increase in lignin content, a well-known process in
246 disease resistance.

247

248 Eudicot plant-specific GIPCs appeared to act as NLP (necrosis and ethylene-inducing
249 peptide 1-like protein) cytolysin receptors (Lenarcic *et al.*, 2017). NLP are produced by
250 bacterial, fungal, and oomycete plant pathogens. Monocots did not develop necrotic lesions
251 upon challenge with NLP. The difference between the two clades resides in the length of
252 terminal hexose residues in GIPCs (two for eudicots and three for monocots). The GIPC
253 sugar moiety is exposed at the surface of the plasma membrane and is thus accessible to
254 NLP binding. The presence of a third hexose unit in monocots impeded NLP insertion into
255 the plasma membrane. The structural and molecular consequences for the plasma
256 membrane that could occur downstream of this recognition requires further study. These
257 studies demonstrate that GIPC glycosylation and the identity of the glycan headgroup are
258 important for the plant immune response.

259

260 **Sphingolipids as signaling messengers in abiotic stress**

261 The sessile nature of plants has driven them to develop a myriad of strategies to resist cell
262 damage. Abiotic stress affects plant growth and development, resulting in loss of vigor and
263 ultimately death. The altered physical and chemical composition of cell membranes under
264 temperature, salt stress or hypoxia is a problem the plant must manage. As a major
265 component of plasma membranes, sphingolipids are significant in mitigating abiotic stress,
266 both in plasma membrane remodelling, and as signal transduction molecules (Ali *et al.*,
267 2018). A summary of the available data on the enzymes and genes of the sphingolipid
268 pathway involved in response to both abiotic and biotic stress is presented in Table 1.

269

270 *Temperature stress*

271 Sphingolipids are involved in cold acclimation as structural components of membranes and
272 also as signaling molecules. In Arabidopsis WT plants, low temperature triggers an
273 accumulation of total sphingolipids, whereas the ratio of unsaturated LCBs is not increased
274 by low temperature (Nagano *et al.*, 2014). This suggests that sphingolipids containing
275 unsaturated LCBs are potential candidates for natural resistance to low temperatures but not
276 for induced tolerance to cold. The cell death suppressor AtBI-1 is involved in sphingolipid
277 synthesis in response to cold by interacting with AtSLD1, AtFAH1, AtSBH2 (a LCB C-4
278 hydroxylase) and AtADS2 (acyl lipid desaturase 2) through Arabidopsis cytochrome *b*₅
279 (Nagano *et al.*, 2014). Moreover, chilling induced a decrease in LCB production (especially
280 t18:1) (Guillas *et al.*, 2013). An Arabidopsis mutant exhibiting low levels of nitric oxide (NO)
281 displayed an accumulation of t18:1. A rapid and transient production of t18:0-P and
282 ceramide-phosphates is induced by cold. This accumulation was negatively regulated by NO
283 (Cantrel *et al.*, 2011) and was specifically impaired in *lcbk2* (but not in *lcbk1*) or *acd5*
284 mutants, respectively (Dutilleul *et al.*, 2012; Dutilleul *et al.*, 2015). Whether NO is able to
285 directly regulate enzymes involved in LCB/LCB-P and Cer/Cer-P rheostat or their substrate
286 availability is still unknown. *lcbk2* displayed a constitutive activation of a cold-responsive
287 MAPK, AtMPK6, at 22°C. AtMPK6 activation was also stimulated by t18:0-P treatment
288 (Dutilleul *et al.*, 2012). The expression of some cold-responsive genes and phenotypical cold
289 responses were impaired in *lcbk2* mutant but not in *acd5*. In addition, *acd5* seed germination
290 was hypersensitive to cold and abscisic acid (ABA), however, gibberellic acid (GA) treatment
291 reverted the *acd5* germination phenotype at 4°C. Germination is regulated by ABA and GA,
292 two hormones that function antagonistically. This suggests that defects in ABA/GA balance
293 and CerK activity could be responsible for *acd5* seed hypersensitivity (Dutilleul *et al.*, 2015).
294 Thus, some responses are regulated by phosphorylated sphingolipids, ABA and NO
295 signaling during cold stress. Recent data reported a role of LCBK1 in Arabidopsis freezing
296 tolerance (Huang *et al.*, 2017). Typical responses including osmolyte accumulation, induction
297 of cold- and membrane lipid-related genes occurring during this abiotic stress are all impaired
298 in *lcbk1* mutant. This suggested a fine-tuned regulation in which LCBK1 acts as a signal in
299 response to freezing temperatures and LCBK2 in response to chilling temperatures.

300

301 There are only a small number of studies indicating that sphingolipid metabolism is also
302 involved in heat stress. It was shown that exogenous LCB-phosphate contribute to heat
303 stress tolerance in Arabidopsis cell culture (Alden *et al.*, 2011). Moreover, a recent
304 transcriptome analysis showed that *AtSLD1* expression is significantly decreased in
305 response to a combination of heat wave and drought at ambient and elevated CO₂,
306 mimicking global changes in climate (Zinta *et al.*, 2018).

307

308 *Hypoxia and oxidative stress*

309 Hypoxia leads to an increase in ceramides, hydroxyceramides, GlcCers and GIPCs (Xie *et al.*, 2015a; Xie *et al.*, 2015b). In hypoxic conditions, GIPCs are elevated in Arabidopsis and
310 *al.*, 2015a; Xie *et al.*, 2015b). In hypoxic conditions, GIPCs are elevated in Arabidopsis and
311 increased further in *Atacbp3* (acyl-CoA binding protein 3) whereas AtACBP3-overexpressors
312 were hypersensitive to submergence (Xie *et al.*, 2015b; Lung & Chye, 2019). Similarly,
313 reduction of unsaturated VLC-ceramides in *loh1*, *loh2* and *loh3* mutants due to the disruption
314 of ceramide synthase is accompanied by an enhanced sensitivity to dark submergence. The
315 *loh1-1 loh3-1* double mutant displayed a reduction of unsaturated very-long-chain (VLC)-
316 ceramides and impaired tolerance to dark and light submergence. Unsaturated VLC-
317 ceramides are therefore seen as defense molecules for plant tolerance to hypoxia (Xie *et al.*,
318 2015a). The mechanism underlying this tolerance involves the modulation of ethylene
319 signaling. These molecules were shown to interact with constitutive triple response1 (CTR1; a
320 negative regulator in ethylene signaling) and to inhibit its kinase activity (Xie *et al.*, 2015a)
321 and subsequent ethylene signaling. Furthermore, the hypersensitivity of *loh* mutants to dark
322 submergence was rescued by introduction of the *crt1-1* mutation that constitutively induces
323 ethylene response. Overexpression of long-chain base kinase (OsLCBK1) in tobacco led to
324 an increased tolerance to oxidative stress provoked by a treatment with either methyl
325 viologen or H₂O₂, accompanied with an induction of oxidative stress-related gene expression
326 (Zhang *et al.*, 2013). *orm1* amiR-*ORM2* plants exhibited an early senescence phenotype
327 accompanied by ROS production and they displayed higher survival rates to oxidative stress
328 (Li *et al.*, 2016). Measurement of sphingolipids showed an increase in LCBs and ceramides
329 and an active vesicular transport that could contribute to the onset of the senescence
330 phenotype and the resistance to oxidative stress. A homolog of human ceramidase, the
331 neutral ceramidase nCer1, was recently characterized. *ncer1* Arabidopsis plants accumulated
332 hydroxyceramides and were more sensitive to oxidative stress. Conversely, *nCer1* over-
333 expressing plants were more tolerant to oxidative stress (Li *et al.*, 2015). Loss of AtACER,
334 encoding an alkaline ceramidase, inhibited autophagy and its overexpression stimulated
335 autophagy under oxidative stress (Zheng *et al.*, 2018). *Atacer* mutant is highly sensitive to
336 oxidative stress whereas the complementation line showed a similar tolerance to this stress
337 as the WT (Zheng *et al.*, 2018). This result suggests that AtACER improves adaptation to
338 oxidative stress by regulating autophagy.

339

340 *Salt stress*

341 During the early stage of salt stress in *Carex rigescens*, an iTRAQ-based proteome study
342 showed a reduction of the enzyme that catalyzes the second step of the biosynthesis of

343 phytosphingosine, 3-ketosphingosine reductase (KDSR) (Li *et al.*, 2017). Based on work
344 performed in yeast where 3-ketosphinganine reductase suppressed Ca²⁺ sensitivity (Beeler
345 *et al.*, 1998), the authors hypothesized that KDSR acts as a suppressor of the calcium signal
346 during a salt stress. Seeds of *Atgint1* (glucosamine inositolphosphorylceramide transferase1,
347 responsible for the glycosylation of some GIPCs) mutants displayed a higher germination
348 rate than WT in response to salt stress, though this difference disappeared at higher salt
349 concentration (Ishikawa *et al.*, 2018). The *Atacer* mutant and *AtACER* RNAi lines displayed
350 high ceramide levels but reduced LCBs due to a disruption of an alkaline ceramidase gene
351 (Wu *et al.*, 2015a). Whereas these plants showed increased sensitivity to salinity, *AtACER*
352 overexpression led to an increased tolerance to such a stress, highlighting the involvement of
353 ceramides in response to salt stress. More precisely, it has recently been shown that
354 *AtACER* regulates autophagy induced by high salt stress (Zheng *et al.*, 2018).
355 Overexpression of a rice S1P (sphingosine-1-phosphate) lyase gene in tobacco led to a
356 decrease in tolerance to salt and changes in salt-stress related genes (Zhang *et al.*, 2012). In
357 contrast, overexpression of *OsLCBK1* in tobacco plants triggered no alteration in expression
358 of salt stress-related genes or tolerance/sensitivity phenotype compared to control plants in
359 response to salt stress (Zhang *et al.*, 2013), suggesting that this enzyme is not involved in
360 salt stress responses in rice. Bioinformatic analysis supported the hypothesis that there are
361 at least two *OsLCBKs* (Zhang *et al.*, 2013). No sphingolipidomic analysis has been
362 performed to reveal how the LCB content could vary between these two over-expressing
363 plants. Previously published papers suggested that the sphingolipid metabolism could be
364 adjusted, so that length chain, concentration, and threshold are important for the sphingolipid
365 function.

366

367 *Interplay with ABA signaling pathway*

368 ABA has a key function in cold/drought stress responses. Pioneering work on sphingolipids
369 showed that d18:1-P and t18:0-P were rapidly induced by drought and were involved in ABA
370 signaling pathway to control guard cell turgor and thus stomatal aperture (Ng *et al.*, 2001;
371 Coursol *et al.*, 2003; Coursol *et al.*, 2005). This sphingolipid signaling pathway involved Ca²⁺
372 mobilization, modification of ion channel activity, and heterotrimeric G-protein. Consistent
373 with this, *AtLCBK1* was reported to be induced by low-humidity or ABA treatments (Imai &
374 Nishiura, 2005). Moreover, ABA also induces the accumulation of several LCB-Ps (Guo *et al.*
375 *et al.*, 2012). SPHK1 is an enzyme that phosphorylates d18:1 and t18:0. Stomata of SPHK1-OE
376 and of *Atspp1* mutant (which accumulates d18:1-P) displayed a higher sensitivity than WT to

377 ABA (Worrall *et al.*, 2008; Nakagawa *et al.*, 2012). Thus, LCB-P content regulated by LCB
378 kinases and phosphatases play a key role in the ABA signaling pathway.

379

380 *Interplay with phospholipid metabolism*

381 Similar to sphingolipids, phosphatidic acid (PA) is considered as a lipid messenger involved
382 in plant response to both biotic and abiotic stress. Like sphingolipids, PA interacts with MPK6
383 during salt stress response in Arabidopsis (Yu *et al.*, 2010) and NADPH oxidase to regulate
384 ROS production during ABA-regulated stomatal closure (Zhang *et al.*, 2009). The PA
385 biosynthetic pathway responds to temperature and salt stress and interacts with sphingosine
386 kinases (Guo *et al.*, 2011). Moreover, addition of exogenous PA induced LCB-P production
387 and LCB-P levels are diminished in *pldα1* in response to ABA (Guo *et al.*, 2012). Over-
388 expression of sphingosine kinase increased PA accumulation. Altogether, the cross-talk
389 between PA and sphingolipids should be a critical point to coordinate a stress response that
390 needs to be elucidated (Fig. 3) (Guo & Wang, 2012; Ng & Coursol, 2012). DAG is a by-
391 product of the IPC synthase and is known to promote stomatal opening (Lee & Assmann,
392 1991; Peters *et al.*, 2010). Although there is no direct evidence for a relationship between
393 sphingolipids and DAG (Fig. 3), lipidome remodeling under stress could yet prove a link.

394

395 **Signaling messengers in biotic stress**

396 Biotic stress caused by plant pathogens and insects are a major threat to both plant survival
397 and productivity. Plants have developed a complex set of defenses when challenged by
398 pathogens. A successful innate immune response depends on the capability of the plant to
399 recognize its invader and then to translate the different stimuli to an adaptive response. As
400 structural plasma membrane components, sphingolipids are important molecules on the front
401 line of pathogen recognition. Sphingolipid disruption also has an impact on PCD and
402 accumulation of several well-known defense molecules (such as ROS, MAPK, and
403 hormones) and sphingolipids thus act as mediators in the defense signaling cascade.

404

405 Very recently, metabolomic profiling identified changes in the sphingolipid pool after exposure
406 to biotic stress. *Xanthomonas campestris* pv. *campestris* infection on *Brassica oleracea*
407 triggered dynamic changes in sphingolipid metabolism including a reduction in the levels of
408 ceramide N-palmitoylsphinganine (Tortosa *et al.*, 2018). Treatment of tomato fruit with the β-
409 aminobutyric acid elicitor increased the detected levels of ceramide phosphatidylinositol

410 (Wilkinson *et al.*, 2017). These metabolomic studies suggested that biotic stresses could
411 impact sphingolipid metabolism.

412

413 *Interplay with SA signaling pathway*

414 Genetic and biochemical data suggests that sphingolipids are involved in the regulation of SA
415 levels. Several mutants with altered sphingolipid metabolism displayed higher SA content
416 and activation of SA-dependent responses. Conversely, both SA and its analogue
417 benzothiadiazole affected sphingolipid metabolism (Shi *et al.*, 2015). The Arabidopsis *fah1/2*
418 mutant displayed SA accumulation in addition to an increase in ceramides but moderate
419 changes in LCB accumulation (König *et al.*, 2012). This suggests that elevated ceramide
420 levels lead to an increase in salicylate levels. In contrast, the Arabidopsis *loh1* mutant
421 displayed an accumulation of C16-ceramides but no changes in SA levels (Ternes *et al.*,
422 2011). This discrepancy suggests the sphingolipid trigger for SA accumulation may be more
423 complicated than initially expected. It is noteworthy that these mutants displayed other
424 changes in sphingolipid homeostasis (for example *fah1/2* also shows a decrease in
425 glucosylceramides) that maybe have previously been overlooked. The induction of SA could
426 thus be due to alterations in sphingolipid classes other than LCBs or ceramides. The link
427 between sphingolipid metabolism and SA may rely on MPK6, ROS/NO and/or calcium
428 accumulation but this is still unclear (Sanchez-Rangel *et al.*, 2015). For example,
429 overexpression of LCBK1 in tobacco cell culture triggered the accumulation of ROS in
430 response to cryptogein. Loss of LCBK activity by using inhibitors resulted in a decrease in
431 ROS production but had no effect on cytosolic calcium influx in elicited tobacco cells (Coursol
432 *et al.*, 2015).

433

434 In conjunction with activation of the SA pathway, several studies revealed that plants
435 disrupted in sphingolipid biosynthesis are also affected in their ability to tolerate biotrophic
436 pathogens. Whereas SA is considered essential for resistance to biotrophic and
437 hemibiotrophic pathogens, it has been demonstrated that jasmonic acid (JA) and ethylene
438 (ET) signaling pathways are important for resistance to necrotrophic pathogens in
439 Arabidopsis (Thomma *et al.*, 2001; Glazebrook, 2005). In Arabidopsis, it is now
440 acknowledged that SA has a reciprocal antagonistic effect on JA signaling (Glazebrook,
441 2005). Using *orm1* amiR-*ORM2* plants, Li *et al.* (2016) demonstrated that the loss of ORM
442 function triggered a constitutive induction of SA-dependent gene and a tolerance to
443 *Pseudomonas syringae* strain DG3 compared to WT plants. *acd5*, *erh1* (enhancing RPW8-
444 mediated HR-like cell death) and *fah1/2* mutants also exhibited a constitutive activation of SA
445 pathway and enhanced resistance to powdery mildew. However, they had a similar
446 phenotype to WT after challenge with the hemibiotrophic pathogens *P. syringae* pv.

447 *maculicola* or *Verticillium longisporum* (Wang *et al.*, 2008; König *et al.*, 2012). Similarly,
448 overexpression of *OsSPL1* in tobacco dramatically reduced SA-dependent gene expression
449 and increased susceptibility to *P. syringae* pv. *tabaci*. Conversely, *PDF1.2*, a JA-dependent
450 gene, expression is slightly enhanced (Zhang *et al.*, 2014). SA-dependent pathogenesis-
451 related (*PR*) gene expressions were constitutively lower in *Atacer-1* plants compared to WT
452 plants. This profile was similar but enhanced when these plants were infected by the *P.*
453 *syringae* strain DG3. As a consequence, *Atacer-1* plants were found more susceptible to the
454 biotrophic *P. syringae* strain DG3 (Wu *et al.*, 2015a). In the light of the antagonistic
455 relationship between SA and JA, it would be interesting to analyze SA and JA levels
456 alongside JA-responsive genes in *Atacer-1* plants.

457

458 Few studies have analyzed the role of sphingolipids during plant/necrotrophic pathogen
459 interaction. Tobacco plants where SPT was silenced accumulated SA, constitutively
460 expressed SA-induced genes and showed an increased susceptibility to the necrotrophic
461 fungus *Alternaria alternata* f. sp. *lycopersici* (Rivas-San Vicente *et al.*, 2013). Similarly, the
462 SA accumulating *acd5* showed increased susceptibility to *B. cinerea* (Bi *et al.*, 2014).

463

464 The role of sphingolipid metabolism in response to herbivory has been analyzed (Begum *et al.*
465 *et al.*, 2016). Overexpression of *OsLCB2a* in Arabidopsis led to the accumulation of LCB and
466 ceramides compared to WT. These transgenic plants also displayed increased callose and
467 wax deposition, an induction of SA- and camalexin-dependent genes but a reduction of JA-
468 related genes, and inhibited aphid infestation (Begum *et al.*, 2016).

469

470 *Interplay with JA signaling pathway*

471 The *Atdpl1* mutant displayed a sensitivity towards the hemibiotrophic bacterium
472 *Pseudomonas syringae* pv. *tomato* but a tolerance when infected by the necrotrophic fungus
473 *Botrytis cinerea* (Magnin-Robert *et al.*, 2015). However, SA levels were similar or even
474 reduced compared to WT whereas JA levels and JA-dependent gene expression were higher
475 in the *Atdpl1* infected mutant. This suggested a link between the sphingolipid and JA
476 pathway. By using *SPHK1* overexpressing plants, SA production was enhanced in response
477 to FB1 treatment. Conversely *SPHK1*-KD plants displayed an increase in JA related
478 transcripts and metabolites (Qin *et al.*, 2017). Thus, it was suggested that the balance
479 between LCBs and LCB-Ps modulated by the activity of *SPHK1* acted as a signal upstream
480 of the SA/JA signaling pathways during FB1-induced cell death (Qin *et al.*, 2017).

481

482 *Interplay with ethylene signaling pathway*

483 It was recently shown that sphingolipid metabolism has connections with not only SA and JA
484 pathways but also with ethylene signaling. Ethylene or its precursor (1-aminocyclopropane
485 carboxylic acid) inhibits sphingolipid biosynthesis. Mutants disturbed in ethylene biosynthesis
486 or signaling displayed constitutive modifications in sphingolipid content (Wu *et al.*, 2015b).
487 For example, *ctr1-1* mutants, which have enhanced ethylene signaling, contained lower
488 levels of ceramides and hydroxyceramides compared to WT. Some constitutive ethylene
489 response mutants displayed a higher tolerance to FB1 and mutants deficient in ethylene
490 signaling exhibited more sensitivity to FB1, showing that enhanced ethylene signaling
491 rescues FB1-induced cell death.

492

493 **Conclusions and future directions**

494

495 In the last few decades we have learned much about the role of sphingolipids during the plant
496 stress response. Functional analyses have demonstrated that sphingolipids are involved in
497 the response to environmental cues. The role of sphingolipids during PCD is well studied.
498 Significant progress has been made but the precise identity of sphingolipids involved in this
499 process is not clearly defined. It is clear that PCD is tightly regulated and further consideration
500 should be given to the different stresses triggering PCD and also the plant species in
501 question. The plasma membrane mediates contact with the environment and is the likely
502 initial source of signal transduction. Recent evidence has shown that GIPC glycosylation
503 involved different regulation processes in the plasma membrane. The composition, the
504 distribution and the dynamic association of sphingolipids are thus of high importance for the
505 plasma membrane function. It is essential to unravel the dynamic association between
506 sphingolipids, plasma membrane lipids and proteins in order to better understand the
507 recognition step of the immune response. While a body of evidence has revealed functions
508 for LCBs/LCB-Ps, ceramides and GIPCs, the roles of GlcCers in plants have yet to be fully
509 investigated, other than the observation that they are essential for normal plant growth and
510 development. The relationship between sphingolipids and SA is long acknowledged and
511 recent studies showed interconnections with other defense signaling pathways such as JA
512 and ethylene. The regulation of stomatal aperture is of crucial importance during plant
513 defense responses especially in response to foliar pathogens. ABA-mediated stomatal
514 closure inhibits pathogen penetration to the apoplast. Since sphingolipid signaling pathway
515 have some interconnections during this process in response to drought stress, the
516 relationship between sphingolipids and ABA in response to foliar pathogens remains to be
517 elucidated.

518

519 Despite the range of different structures of sphingolipids and differing physical properties they
520 exhibit, understanding of sphingolipid regulation and function is not comprehensive. The
521 interactions with other cellular lipids are also yet to be fully resolved but there are known
522 relationships with several other lipid classes. The wider lipidome is subject to remodeling
523 when the plant is under stress and it is likely that sphingolipids form part of a coordinated
524 response. The mechanisms for action and whether sphingolipids regulate stress responsive
525 gene expression or are themselves regulated by stress responsive transcription factors are
526 not yet fully understood. There is still a gap in understanding the role of sphingolipids in the
527 plant stress response, but the advent of genome editing technology opens the possibility to
528 develop crops with a greater ability to tolerate stress based on the manipulation of their
529 sphingolipid biosynthetic pathway.

530

531

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538

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829

830 Figure legends

831 **Fig. 1** Schematic representation of the sphingolipid biosynthetic pathway in plants. 3-KSR, 3-
832 Ketosphinganine Reductase; ACD5, Accelerated Cell Death 5; ACER, Alkaline Ceramidase;
833 Cer, Ceramide; Ceramide-P, Ceramide-Phosphate; coA, CoenzymeA; DAG, Diacylglycerol;
834 DPL1, Dihydro sphingosine-Phosphate Lyase; ERH1, Enhancing RPW8-Mediated HR-like
835 Cell Death; FA, Fatty Acid; FAH, Fatty Acid Hydroxylase; GC, Glucosylceramide; GINT1,
836 Glucosamine Inositolphosphorylceramide Transferase 1; GIPC, Glycosyl Inositol Phospho
837 Ceramide; GMT1, GIPC Mannosyl-Transferase 1; GONST1, Golgi Localized Nucleotide
838 Sugar Transporter 1; IPCS, Inositol Phosphorylceramide Synthase; IPUT, Inositol
839 Phosphorylceramide Glucuronosyltransferase 1; LCB1,2, Subunit of Serine
840 Palmitoyltransferase 1 and 2; LCB, Long-Chain Base; LCB-P, Long-Chain Base Phosphate;
841 LOH, Lag One Homolog; NCER, Neutral Ceramidase; ORM, Orosomucoid-like Protein; PI,
842 Phosphoinositol; SBH, Sphingoid Base Hydroxylase; SL, Sphingolipid; SLD, Sphingolipid Δ8
843 Long-Chain Base Desaturase; SPHK, Sphingosine Kinase; ssSPT, Small Subunit of Serine
844 Palmitoyl Transferase; SPT, Serine Palmitoyl Transferase.

845 **Fig. 2** Sphingolipid rheostat. The equilibrium between ceramides/Long chain bases (LCBs)
846 and ceramide-phosphates (Ceramide-Ps)/LCB-Ps defines cell fate.

847 **Table 1** Enzymes and genes of sphingolipid metabolism involved in response to (a)biotic
848 stress.

849

850 **Supporting Information**

851 **Table S1** Abbreviations used in this review.